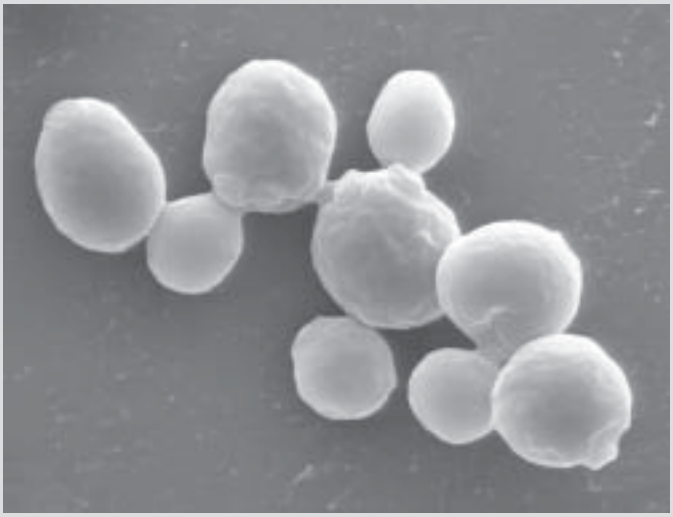


Selective Glycoprotein Exit from Yeast Endoplasmic Reticulum

NETTA FATAL

Institute of Biotechnology
Program in Cellular Biotechnology
Department of Biological and Environmental Sciences
University of Helsinki

Dissertationes Biocentri Viikki Universitatis Helsingiensis

12/2004

Selective glycoprotein exit from yeast endoplasmic reticulum

Netta Fatal

Institute of Biotechnology,
Department of Biological and Environmental Sciences, Division of Biochemistry, and
Viikki Graduate School in Biosciences

University of Helsinki
Finland

ACADEMIC DISSERTATION

To be presented for public criticism, with the permission of
the Faculty of Biosciences of the University of Helsinki, in Auditorium 1041
at the Viikki Biocenter (Viikinkaari 5), Helsinki,
on June 4th, 2004, at 12 noon.

Supervised by
Professor Marja Makarow
Institute of Biotechnology and
Department of Applied Chemistry and Microbiology
University of Helsinki

Reviewed by
Docent Varpu Marjomäki
Department of Biological and Environmental Science
University of Jyväskylä

Docent Johanna Myllyharju
Department of Medical Biochemistry and Molecular Biology
University of Oulu

Opponent
Professor Jaakko Saraste
Department of Biomedicine
University of Bergen
Norway

Kustos
Professor Carl G. Gahmberg
Department of Biological and Environmental Sciences
Division of Biochemistry
University of Helsinki

ISBN 952-10-1839-9
ISBN 952-10-1840-2 (ethesis)
ISSN 1239-9469

Gummerus Kirjapaino Oy
Saarijärvi, 2004

Cover image
Scanning electron micrograph of yeast *Saccharomyces cerevisiae*,
strain SEy2101a (H1) used in studies I and II of this thesis. Picture kindly provided by Eija Jokitalo, Institute of Biotechnology.

To my mother, Masa, and Elia

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following original articles and manuscript, which are referred to in the text by their Roman numerals:

I **Fatal, N.**, Suntio, T., and Makarow, M. 2002. Selective protein exit from yeast endoplasmic reticulum in absence of functional COPII coat component Sec13p. Mol. Biol. Cell 13:4130-4140.

II **Fatal, N.**, Karhinen, L., Jokitalo, E., and Makarow, M. 2004. Active and specific recruitment of a soluble cargo protein for endoplasmic reticulum exit in the absence of functional COPII component Sec24p. J. Cell Sci. 117:1665-1673.

III **Fatal, N.**, Toikkanen, J. H., Hildén, P., Makarow, M., Keränen, S., and Kuismanen, E. A family of BAP31-like proteins has three putative members in *Saccharomyces cerevisiae*, Manuscript.

ABBREVIATIONS

ARF	ADP ribosylation factor
ATP	adenosine triphosphate
CHX	cycloheximide
COP	coat protein
CPY	carboxypeptidase Y
DAB	diaminobenzidine
EM	electron microscopy
EMS	ethyl methanesulfonate
ER	endoplasmic reticulum
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GFP	green fluorescent protein
GMP-PNP	guanylyl imidodiphosphate
GTP	guanosine triphosphate
GTPγS	guanosine 5'-[γ-thio]triphosphate
HRP	horseradish peroxidase
Hsp	heat shock protein
kDa	kiloDalton
ORF	open reading frame
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGD	*Saccharomyces* Genome Database
SNARE	soluble N-methylmaleimide-sensitive factor attachment protein receptor
STAR	Sar1-NH_2-terminal activation recruitment
SUI	Subunit I
VSV G	vesicular stomatitis virus glycoprotein
TMD	transmembrane domain

SUMMARY

Hsp150 and different Hsp150 variant proteins were used to study mechanisms of cargo selection in the first vesicular step of the secretory pathway in yeast *Saccharomyces cerevisiae*. This anterograde step is mediated by vesicles covered with a cytosolic coat called COPII. Transport of Hsp150 from the endoplasmic reticulum (ER) to the Golgi complex was shown not to require functional COPII components Sec13p or Sec24p. However, other COPII proteins were required, indicating that the COPII machinery driving vesicle formation at the ER membrane was not entirely dispensable.

The determinant(s) guiding Hsp150 to the Sec13p- or Sec24p-independent pathways was mapped to the C-terminal domain of Hsp150. While Hsp150 variants lacking the C-terminal domain were secreted in wild-type cells, the domain was shown to be essential for ER exit of Hsp150 in the absence of functional Sec13p or Sec24p. Furthermore, the C-terminal domain was able to recruit a Sec13p- and Sec24p-dependent yeast protein, invertase, into vesicles generated with nonfunctional Sec13p or Sec24p. This suggests that the signal in the C-terminal domain of Hsp150 is an active mediator of ER exit.

A new protein family of BAP31-like proteins was also characterized in this study. The putative family members, identified based on several common structural features, were found in various eukaryotic organisms. The best-characterized member of this family, mammalian BAP31, appears to participate both in apoptosis and in enhancing ER exit of a subset of proteins. The yeast members of this family were shown to affect yeast growth when deleted. However, these alterations in growth rates were not associated with defects in the ER exit of selected yeast proteins.

INTRODUCTION

The ability to transport proteins via the secretory pathway is fundamental to every eukaryotic cell. A continuous, bidirectional flow of proteins through this pathway is required to sustain the functionality of the cell. Proteins entering the pathway include the resident proteins of the organelles of the pathway itself as well as various plasma membrane, cell wall, and extracellular proteins.

The role of the secretory pathway is not limited to transportation of proteins only. As proteins are transported in membraneous containers, in addition to protein transfer, the transfer of lipids must be taken into account. The yeast *Saccharomyces cerevisiae* is a unicellular eukaryote that grows by budding. Thus, increasing the surface area of the cell is not limited by neighboring cells, as in mammalian tissues. One important function of the secretory pathway in yeast, besides protein transport, is therefore to increase the surface area of the growing bud by importing new membrane to the tip of a growing new daughter cell. By contrast, in mammalian cells, the anterograde transport of vesicles has to be counterbalanced with retrograde transport to keep the surface area of the cell constant. Therefore, the balance in membrane traffic can be envisioned to be different in these two cell types. The retrograde transport in yeast is not required for limiting cell surface growth. Instead, the focus of retrograde traffic is more on protein traffic; various proteins transported along the secretory pathway, such as cargo receptors or vesicle fusion factors, are recycled for use several times during their life span.

Over the past three decades, tremendous progress has occurred in uncovering the molecular mechanisms of the secretory pathway. The foundation was laid by George Palade and his coworkers, who unraveled the general scheme of the secretory pathway. By investigating the acinar cells of the pancreas, they showed that newly synthesized secretory proteins are transported through distinct membrane-bound organelles, the endoplasmic reticulum (ER) and the Golgi complex, before arrival at the plasma membrane [reviewed in George Palade's Nobel lecture (Palade, 1975)].

1. Key discoveries in studying the yeast secretory pathway

Access to genome sequences of several eukaryotic organisms has revealed that many of the core biological processes are conserved in evolution. Today, many cell biologists routinely use yeast *S. cerevisiae* as a eukaryotic model.

1.1 Screen for identifying yeast mutants defective in secretion

At the beginning of the 1970s, *S. cerevisiae* was already used for cell cycle studies (see *e.g.* Hartwell, 1991). Yeast was perhaps not the obvious organism for studying protein secretion because the cells are not specialized for secretion and,

consequently, the organelles involved in this function are modest. This, in turn, caused problems in morphological analyses, as some of the organelles (like the Golgi) could not be reliably detected. However, some of the genetic methods, such as generation of conditional mutants, were already at hand. In addition, although a coherent model did not exist, bits of information were available about glycoprotein secretion in yeast (see Novick *et al.*, 1980).

Yeast strains that are defective in protein secretion but nevertheless continue to synthesize new proteins become denser than wild-type cells due to accumulation of secretory proteins inside the cells. This discovery by Novick and Schekman (1979) marked the beginning of a new era of protein secretion studies, where yeast was used as a model.

A study describing the isolation of 23 yeast strains defective in protein secretion due to mutation of a single gene (secretory or *sec* mutants) was a milestone in identifying the yeast secretory pathway (Novick *et al.*, 1980). The set-up of the mutant isolation assay was simple and elegant. Yeast strains were first mutagenized with ethyl methanesulfonate (EMS) or nitrous acid. The cells were then allowed to recover from the mutagenesis at a permissive temperature (25°C)* for 16 hours in a rich medium and then transferred to a restrictive temperature (37°C) for three hours. During this time the cells that were unable to secrete proteins due to a temperature-sensitive mutation in a gene affecting protein secretion became denser. The cells were then fractionated in a Ludox density gradient, a technique developed earlier for separating yeast cells based on their density (Henry *et al.*, 1977). Only now, the gradient was used to enrich putative temperature-sensitive secretion mutants. The densest cells obtained by fractionation were tested for ability to grow at 37°C, and those that did not grow (indicating temperature sensitivity) were tested for secretion of acid phosphatase and invertase. The level of general protein synthesis at 37°C was also measured.

The clones that failed to secrete but were not defective for protein synthesis at 37°C were selected for further analysis. Two haploid mutants of opposite mating type (**a** and α) were crossed to obtain a diploid (**a**/α). If the diploid grew at 37°C, it could be concluded to have one wild-type copy of both mutated genes, with these intact copies suppressing the temperature-sensitive phenotypes detected in haploid parents. Thus, if the diploid grew at a restrictive temperature, the mutations were in different genes and the parental haploids represented different complementation groups. If, however, the diploid remained temperature-sensitive, then the mutations in the original haploids were likely to be in the same gene; no wild-type copy of the respective gene ensuring growth at elevated temperatures was provided by either haploid. In that case, the haploids represented the same complementation group.

The researchers obtained 23 different complementation groups (*sec1-sec23* mutant strains) that fulfilled the following criteria: (1) they failed to secrete acid phosphatase and invertase at 37°C, (2) protein synthesis was not compromised at

* Optimal growth temperature for yeast cells is 20-30°C, but yeast can grow at 38-39°C. Yeast strains with no secretory mutations are usually grown at 30°C.

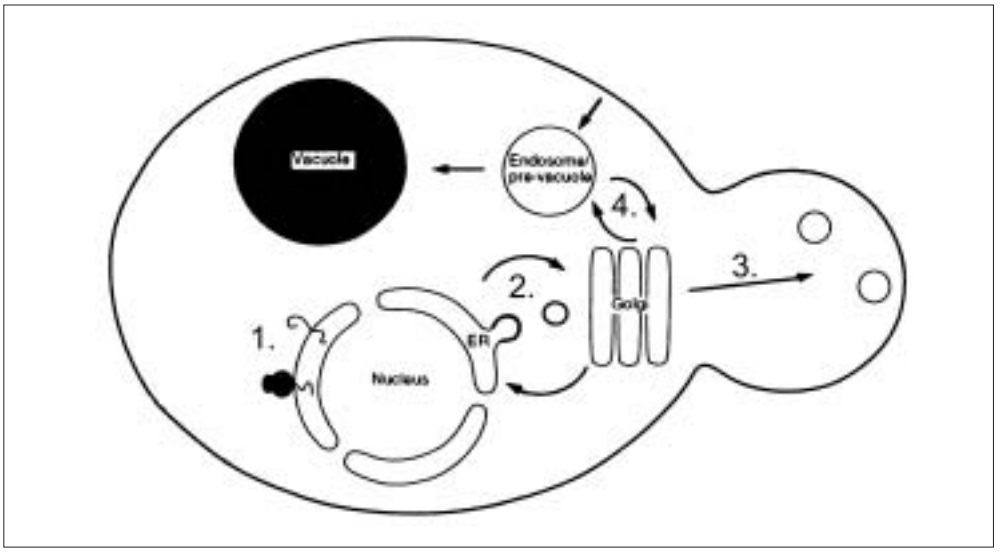


Figure 1. Overview of the secretory pathway in yeast. (1.) Newly synthesized secretory proteins are translocated into the lumen or membrane of the endoplasmic reticulum (ER). In the ER, proteins acquire correct conformation prior to their exit from the compartment. The N-linked core oligosaccharides as well as the first mannose of the O-linked glycan chains are added in the ER. Only correctly folded and modified proteins are allowed to exit the ER. (2.) From the ER, proteins are transported to the Golgi complex, where N- and O-linked glycan chains are elongated by addition of mannoses. Some proteins are specifically processed by proteases in the *trans*-Golgi. From the *trans*-Golgi, proteins are transported to the plasma membrane, cell wall, or culture medium (3.), or to vacuole (4.) *via* prevacuolar compartments. Vesicular traffic between organelles is mostly bidirectional. Picture modified from Kaiser *et al.* (1997).

37°C, (3) the mutations were recessive (*i.e.* heterozygous diploids showed a wild-type phenotype), (4) cell division stopped rapidly after a shift to 37°C, and (5) the mutations were reversible after a three-hour incubation at a restrictive temperature.

Further support that the *sec* mutants indeed represented genes required for protein secretion was obtained from electron microscopic analyses, which revealed a temperature-dependent accumulation of various secretory organelles (Novick *et al.*, 1980). Figure 1 shows the main steps in the yeast secretory pathway.

1.2 Cell-free protein transport from the ER to the Golgi complex

The conditional secretion mutants of yeast could be used for *in vivo* investigations. However, *in vivo* studies depend on living cells, which sets limitations on the experiments that can be performed. To overcome the dependence on live cells, *in vitro* assays for protein transport studies were developed.

At the beginning of the 1980s, intra-Golgi transport was reconstructed in a mammalian cell-free system (Fries and Rothman, 1980; Balch *et al.*, 1984). By

combining Golgi membranes prepared from two populations of mammalian cells and incubating them at 37°C (optimal for this system) in the presence of cytosol and ATP, successfull reconstitution of vesicle-mediated transport between two Golgi cisternae was obtained (Balch *et al.*, 1984). The "donor" membrane in this assay contained an N-glycosylated vesicular stomatitis virus glycoprotein (VSV G) as a marker protein, and the "acceptor" membrane a specific glycosyltransferase that was absent from the cells providing the donor cisternae. Transfer of proteins between the two membranes was verified by analyzing processing of the marker protein by the transferase (Balch *et al.*, 1984).

In yeast, an *in vitro* assay was developed to monitor not the protein traffic between two Golgi cisternae but rather the anterograde transport from the ER to the Golgi. The reason for this was practical: several conditional yeast mutants were available that affected this particular step, and also, the Golgi arrival of a marker protein could be conveniently determined from outer-chain carbohydrate modifications. Two laboratories independently developed almost identical yeast cell-free assays (Baker *et al.*, 1988; Ruohola *et al.*, 1988).

Baker *et al.* (1988) used lysed yeast cells as a source of ER membranes, and radioactively labeled prepro-α-factor (yeast pheromone, a soluble secretory protein with N-glycosylation sites) as a marker protein. The signal peptide of α-factor (pre-) results in the protein being posttranslationally translocated into the lumen of the ER (Ng *et al.*, 1996; Rapoport *et al.*, 1996). The researchers were concerned that the use of a wild-type strain would result in undetectable signals in the assay if the membranes were prepared after the translocation step. Therefore, the use of an exogenous marker protein in high concentrations combined with efficient posttranslational translocation in already lysed cells was adopted.

To be able to examine the requirements of the transport step only, separating the events of translocation and transport was important. This was achieved by manipulating the incubation temperatures; translocation was efficient already at 10°C, whereas transport required incubation at 20°C. As a result, the membranes could be washed after the translocation step. Thereafter, addition of fresh cytosol and an energy-generating system initiated the transport step.

Arrival in the Golgi was verified from the outer-chain modification of the N-glycans of the reporter protein. Typically, 25-30% of the pro-α-factor received N-glycan extensions and were thus transported to the Golgi (Baker *et al.*, 1988).

The main difference in the *in vitro* assay developed by Ruohola *et al.* (1988) was that by varying incubation times rather than temperatures they were able to separate translocation and transport steps.

1.2.1 Vesicle budding assay

Further dissection of the steps between ER and Golgi transport enabled the researchers to study the requirements of vesicle budding, targeting, and fusion independently of each other (Rexach and Schekman, 1991). As this assay will be referred to many times hereafter, I will briefly explain its underlying.

First, yeast membranes are prepared either from a wild-type strain or from a

strain with a temperature-sensitive mutation. The radioactively labeled prepro-α-factor is then allowed to translocate into the lumen of the ER of these membranes. Before use, the membranes are washed with 2.5 M urea to remove all peripheral membrane proteins. The cytosol used in the assay is the 100,000 *g* supernatant fraction of yeast lysates. Today, purified COPII proteins are often used instead of cytosol.

When membranes, purified proteins (or cytosol), nucleotides (GTP or a nonhydrolyzable GTP analog GMP-PNP or GTPγS), and possibly an ATP regeneration system are incubated at physiological temperatures, the supplemented COPII proteins support generation of ER-derived vesicles from the membranes. Since large ER membranes sediment faster than small ER-derived vesicles, the formation of vesicles can be monitored by measuring the amount of radioactivity in a medium-speed supernatant (containing vesicles with labeled pro-α-factor).

These *in vitro* reactions have been invaluable in purification and functional analyses of several proteins participating in the early stages of the secretory pathway (*e.g.* Barlowe *et al.*, 1994; Miller *et al.*, 2002).

2. Proteins involved in formation of ER-derived vesicles

Proteins are transported in a directed fashion from a donor organelle to an acceptor organelle. The formation of the transport unit, often a vesicle, is tightly regulated by cytosolic coat proteins. These proteins are recruited to the membrane where they initiate the formation of the bud, select the cargo for the growing bud, and eventually coat the whole vesicle as it pinches off the membrane. Thereafter, the coat is shed, enabling vesicle targeting and fusion with the acceptor membrane.

The first donor compartment in the secretory pathway is the ER. From there, proteins are transported in vesicles to the first acceptor compartment, the *cis*-face of the Golgi complex. Development of the yeast *in vitro* assays enabled the characterization of the proteins involved in the vesicle formation event at the ER membrane. Using purified ER membranes and purified cytosolic proteins, Barlowe *et al.* (1994) showed that formation of a transport vesicle requires, in addition to membranes, only five cytosolic proteins: a small GTPase Sar1p, and two heteromeric complexes, the Sec23/24p-complex, and the Sec13/31p-complex. The energy requirements were fulfilled with a guanine nucleotide (GTP or its nonhydrolyzable analog GTPγS or GMP-PNP). The coat formed from these components was designated COPII (Barlowe *et al.*, 1994).

Retrograde traffic from the Golgi back to the ER is mediated by another cytosolic coat, COPI (Orci *et al.*, 1986). As in the case of COPII, a small GTPase (Arf1p) is required to initiate the bud formation. The coat itself is formed from preassembled coatomers, complexes of seven proteins (reviewed in Gaynor *et al.*, 1998). A general overview of the bidirectional transport between these two organelles is presented in Figure 2.

COPI and COPII components are highly conserved between yeast and

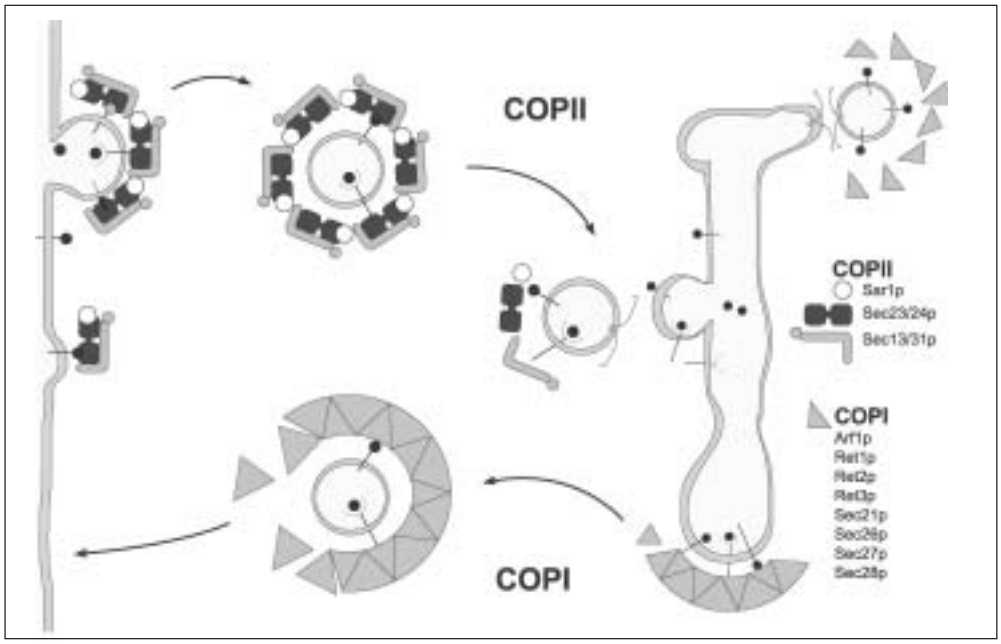


Figure 2. A schematic presentation of the bidirectional traffic between the ER and the Golgi complex. COPII-coated vesicles mediate the anterograde ER-to-Golgi transport, whereas COPI vesicles are responsible for the retrograde traffic from the Golgi back to the ER. Shedding of the coats is required prior to fusion with the acceptor membrane.

mammalian cells. COPI was first identified *in vitro* in Chinese hamster ovary cells by electron microscopic (EM) investigation (Orci *et al.*, 1986). The seven subunits of mammalian coatomer, α-, β-, β'- ,γ-, δ-, ε-, and ζ-COP (Duden *et al.*, 1991; Waters *et al.*, 1991; Stenbeck *et al.*, 1993) as well as the small GTPase, ADP-ribosylation factor (ARF) (Donaldson *et al.*, 1992), were described a few years later. The existence of a similar protein complex in yeast was suggested when the yeast coatomer was purified, and it was shown that one member of this protein complex cross-reacted with antibodies raised against the mammalian β-COP (Hosobuchi *et al.*, 1992). To date, all genes encoding yeast COPI components have been discovered (Gaynor *et al.*, 1998). The discovery of the COPII coat originated from genetic and biochemical studies in yeast (Hicke and Schekman, 1989; Kaiser and Schekman, 1990). Existence of a homologous system in higher eukaryotes was first implicated by detection of a protein that cross-reacts with antibodies specific for yeast Sec23p (Orci *et al.*, 1991), a member of the COPII coat (Barlowe *et al.*, 1994). Thereafter, the genes encoding all mammalian COPII proteins have been cloned (Wadhwa *et al.*, 1993; Kuge *et al.*, 1994; Swaroop *et al.*, 1994; Shaywitz *et al.*, 1995; Paccaud *et al.*, 1996; Pagano *et al.*, 1999; Shugrue *et al.*, 1999; Tang *et al.*, 1999). It is now becoming apparent

that the COPI/COPII machinery is conserved in all eukaryotic organisms; genes coding for the coat components have been isolated for instance from plants (d'Enfert *et al.*, 1992; Kim *et al.*, 1997; Movafeghi *et al.*, 1999), fission yeast (d'Enfert *et al.*, 1992; Payne *et al.*, 2000), and even from *Plasmodium falciparum*, a parasite causing malaria (Albano *et al.*, 1999; Adisa *et al.*, 2002).

2.1 Sec12p

SEC12 was one of the genes identified in the classical screen by Novick *et al.* (1980). In subsequent genetic (Novick *et al.*, 1981) and biochemical (Stevens *et al.*, 1982) analyses, the gene product was shown to participate in protein transport between the ER and the Golgi complex. Morphological investigation by EM of the *sec12-4* cells incubated at a restrictive temperature revealed accumulation of ER membranes and low amounts of 50-nm vesicles (Kaiser and Schekman, 1990). Other *sec* mutants displaying the same phenotype were *sec13*, *sec16*, and *sec23*. These mutants were categorized as class I mutants affecting formation of a transport vesicle at the ER membrane (Kaiser and Schekman, 1990).

SEC12 was cloned as a single copy suppressor of the *sec12-4* mutation. The gene encodes a 471-amino acid type II integral membrane protein of 70 kDa with 1-2 sites for N-linked glycans (d'Enfert *et al.*, 1991a, 1991b). *In vitro* studies confirmed that Sec12p was essential for the vesicle budding step (Rexach and Schekman, 1991).

Sec12p is the guanine-nucleotide exchange factor (GEF) for Sar1p (see Sections 2.2 and 3.2) that stimulates the exchange of GTP for GDP on Sar1p (Barlowe and Schekman, 1993).

2.1.1 Localization of Sec12p

Sec12p is targeted to the ER membrane (Nakano *et al.*, 1988; Rexach and Schekman, 1991; Nishikawa and Nakano, 1993) by two distinct mechanisms: retention and retrieval. The signals act independently and are alone sufficient to confer ER localization for non-ER proteins (Sato *et al.*, 1996). The cytosolic domain of the protein has an as yet unidentified signal for retention; a chimeric protein in which the cytosolic domain of Sec12p is fused to transmembrane and lumenal domains of a vacuolar type II membrane protein, Dap2p, shows ER localization. The N-glycans of this protein are very slowly modified by *cis*-Golgi transferases, indicating relatively stable retention in the ER (Sato *et al.*, 1996). The second determinant is in the transmembrane domain of Sec12p. This motif is committed more to retrieval than retention, and the function is dependent on Rer1p, an integral membrane protein shown to assist recycling of a subset of ER proteins (Nishikawa and Nakano, 1993; Boehm *et al.*, 1994, 1997; Sato *et al.*, 1995, 1996, 2001).

2.2 Sar1p

SAR1 was cloned as a multicopy suppressor of a temperature-sensitive *sec12* mutant (Nakano *et al.*, 1988; Nakano and Muramatsu, 1989). Early studies indicated that Sar1p was required at the first vesicular step in the secretory pathway: depletion of Sar1p *in vivo* led to

accumulation of ER forms of carboxypeptidase Y (CPY), a soluble vacuolar protease (Stevens *et al.*, 1982) and α-factor (Nakano and Muramatsu, 1989). Furthermore, excess Sar1p resumed *in vitro* vesicle budding from *sec12* membranes as well as protein transport in semi-intact *sec12* cells (d'Enfert *et al.*, 1991b; Oka *et al.*, 1991); at that time *SEC12* was already suggested to encode a protein functioning in a vesicle formation step at the ER (Kaiser and Schekman, 1990). In addition to suppressing the temperature-sensitive phenotype of *sec12-4*, overexpression of *SAR1* also suppressed other COPII mutations *sec16-2* (Nakano and Muramatsu, 1989) and *sec23-1* (Oka and Nakano, 1994).

The *SAR1* gene codes for an essential GTP-binding protein of 190 amino acids/ 21 kDa (Nakano and Muramatsu, 1989; Oka *et al.*, 1991) that is present in the COPII coat (Barlowe *et al.*, 1994; Oka and Nakano, 1994). Sar1p is most closely related to the ARF family of small GTPases in the Ras superfamily (Nakano and Muramatsu, 1989). Similar to all other GTP-binding proteins, Sar1p is the target of two additional proteins that regulate its activity; exchange of GTP for GDP is stimulated by the ER-localized Sec12p (GEF for Sar1p; Barlowe and Schekman, 1993), whereas GTP hydrolysis is activated by one of the other COPII components, Sec23p (GTPase-activating protein, GAP, for Sar1p; Yoshihisa *et al.*, 1993).

2.3 Sec23p

A temperature-sensitive mutant allele of the *SEC23* gene was discovered in the original study by Novick *et al.* (1980) to accumulate ER membranes and later classified as a class I mutant affecting vesicle formation (Kaiser and Schekman, 1990). *SEC23* was cloned as a single copy suppressor of the *sec23-1* mutant (Hicke and Schekman, 1989). The gene encodes a protein of 768 amino acids/84 kDa, an essential component of the COPII coat (Barlowe *et al.*, 1994).

2.3.1 Purification of the Sec23/24p complex

ER-to-Golgi transport cannot be reconstituted *in vitro* if both the donor membranes and cytosol used in the reaction are prepared from a *sec23* mutant. If the cytosol added to the reaction mixture is instead prepared from a *SEC23*$^{+}$ strain, it resumes the transport reaction (Baker *et al.*, 1988). Thus, the *in vitro* assay provided a convenient method for purification of Sec23p activity. Hicke and Schekman (1989) fractionated yeast cytosol of a wild-type strain by gel filtration and tested the ability of each fraction to restore the defect in ER-to-Golgi transport in reactions performed with *sec23* membranes and *sec23* cytosol. The fractions containing the Sec23p activity eluted from the column corresponded to a protein size of approximately 400 kDa. Further characterization revealed that Sec23p eluted as a heteromeric complex with a 105-kDa protein, later designated Sec24p (Hicke *et al.*, 1992).

2.3.2 Sec23p is a Sar1p-specific GTPase-activating protein

Although no sequence similarity to other GTPase-activating proteins (GAPs)

existed, Sec23p turned out to be a GAP specific to Sar1p (Yoshihisa *et al.*, 1993). Again, the *in vitro* assays were highly valuable for discovering the function of Sec23p.

By using the vesicle budding assay (Section 1.2.1) Yoshihisa *et al.* (1993) noted that while addition of excess purified Sec23/24p complex to the reaction did not affect the formation of vesicles, addition of excess monomeric Sec23p abolished vesicle production. This effect could be overcome by adding excess purified Sar1p, indicating an interaction between these two proteins. Although Sec23p did not resemble any known GEF or GAP proteins, the Sec23/24p complex or the monomeric Sec23p alone could stimulate GTP hydrolysis on Sar1p 10- to 15-fold (Yoshihisa *et al.*, 1993). The action was specific to Sar1p; no stimulation of GTP hydrolysis by two other known small GTPases, human ARF1 and yeast Ypt1p, was observed. The GTP hydrolysis assay performed with purified proteins confirmed that the interaction between Sar1p and Sec23p is direct (Yoshihisa *et al.*, 1993).

2.4 Sec24p

Sec24p was first identified when the Sec23p-containing large protein complex was analyzed; the complex consisted of two proteins, Sec23p and "p105", the latter name reflecting the electrophoretic mobility of the protein (Hicke *et al.*, 1992). Antibodies raised against the p105 partner inhibited budding of ER-derived vesicles *in vitro*, similarly to Sec23p-specific antibodies. This indicated that the novel protein was also involved in ER-to-Golgi traffic (Hicke *et al.*, 1992).

The *SEC24* gene was first cloned by complementation of a temperature-sensitive *sec24-1* mutation (Gimeno *et al.*, 1996). The same gene was also isolated from a yeast genomic library by screening the library with PCR fragments that had been designed based on information obtained from peptide fragments of trypsin-digested p105 (Kurihara *et al.*, 2000). The gene encodes a 926-amino acid protein with a calculated molecular weight of 103 kDa.

2.4.1 Sec24p is essential for vesicle budding

The *sec24* mutants block the ER-to-Golgi transport of proteins to various degrees. Peng *et al*. (2000) generated temperature-sensitive mutant alleles of *SEC24* by mutagenizing a plasmid containing the wild-type *SEC24* with hydroxylamine and then replacing the chromosomal *SEC24* gene with one of the mutant alleles. All three mutations obtained, *sec24-11*, *sec24-13*, and *sec24-16*, showed only mild effects in maturation of CPY and Gas1p, a glycosylphosphatidylinositol (GPI)-linked plasma membrane protein (Nuoffer *et al.*, 1991). The mutations in these three alleles are within the first 350 amino acids of Sec24p (Peng *et al.*, 2000). The *sec24-11* mutation appears to be in the region interacting with Sec23p, but it is not one of the amino acids required for binding (Bi *et al.*, 2002). The other two mutations, *sec24-13* and *sec24-16*, are closer to the amino terminus, the function of which remains unclear.

A *sec24-20* mutation, by contrast, which had been generated by EMS mutagenization (Kimata *et al.*, 1999), showed a complete block in ER exit of

CPY at a restrictive temperature (Higashio *et al.*, 2000). The *sec24-1* mutation also imposed a tight secretion block for CPY and invertase at a restrictive temperature. However, it did not hinder a soluble glycoprotein, Hsp150, from entering the transport vesicles (Article II of this thesis; see Results and Discussion).

Mapping the mutations in *sec24-20* and *sec24-1* alleles would probably give insight into the mechanism of the ER exit block caused by the nonfunctional Sec24p at the molecular level.

2.5 Sec13p

Sec13p is a hydrophilic protein of 297 amino acids with an apparent molecular weight of 33 kDa. The gene was isolated by screening a yeast genomic library for complementation of the *sec13-1* growth defect at a restrictive temperature (Pryer *et al.*, 1993). Sec13p is composed almost entirely of six WD repeats. This motif can be found in a large selection of proteins with no apparent similarities in function in both eukaryotes and prokaryotes. The only common feature is in mediating protein-protein interactions in multi-protein complexes (reviewed in Li and Roberts, 2001).

The crystal structure of one WD protein, the β-subunit of the hetero-trimeric G protein, has been determined (Wall *et al.*, 1995; Sondek *et al.*, 1996). This protein contains seven WD repeats arranged in a ring, with the repeats forming seven blades in a propeller structure. Each repeat folds into a four-stranded antiparallel β-sheet. One "blade" is composed of four antiparallel β-sheets in such a way that three strands originate from one repeat and the fourth is from the previous repeat (Li and Roberts, 2001). Apparently, all WD repeats fold similarly, and thus, Sec13p is a propeller with six blades (Garcia-Higuera *et al.*, 1998).

Some of the mutant alleles of *SEC13* have been mapped. *sec13-1* and *sec13-3* have a S224K substitution (neutral*) in the fifth blade, within the third β-strand. *sec13-4* has a G266A substitution (neutral) in the sixth blade, in a loop region between the second and third β-strand. Finally, the W262R point mutation (disfavored) in *sec13-5* falls into the second β-strand of the sixth blade (calculated from Pryer *et al.*, 1993). All of these mutations cause a temperature-sensitive phenotype in *SEC13*. Both *sec13-1* and *sec13-3* mutations result in a critical reduction of the activity of the protein, whereas the *sec13-4* mutation has only mild effects (Pryer *et al.*, 1993) that might be related to the degree of structural distortion.

Similar to Sec23p-activity, Sec13p-activity was purified from the yeast cytosol by gel filtration. Fractions restoring activity to an *in vitro* budding assay corresponded to an approximately 700-kDa protein, indicating that Sec13p is probably part of a large homo- or heteromeric complex (Pryer *et al.*, 1993). Analysis of the protein complex revealed only one other protein, "p150" (Salama *et al.*, 1993), later designated Sec31p (Salama *et al.*, 1997).

* The information regarding the nature of amino acid substitutions was obtained from http://www.russell.embl-heidelberg.de/aas/aas.html.

2.5.1 Additional functions of Sec13p

The structure of Sec13p, with no motifs other than WD repeats, gives reason to presume that the protein might play a more general role in the cells. Therefore, it was not surprising that Sec13p together with related protein Seh1p were identified as components of a yeast nucleoporin complex (Siniossoglou *et al.*, 1996). Support for this finding was obtained from mammalian cells; the mammalian homolog of Sec13p was also shown to interact with a set of nuclear pore complex proteins (Fontoura *et al.*, 1999).

The function of Sec13p is also important in the later stages of the secretory pathway. Roberg *et al.* (1997) discovered that transport from the *trans*-Golgi to the vacuole of Gap1p, an inducible amino acid permease, was impaired in a *sec13-1* strain. In *SEC13*$^{+}$ cells, Gap1p is transported to the plasma membrane when the yeast cells are grown on a medium containing ammonia as a source of nitrogen. When provided with glutamate, the permease is transported to the vacuole for degradation. However, in *sec13-1* mutants grown in the presence of ammonia, Gap1p was mislocalized to the vacuole even at permissive temperatures (Roberg *et al.*, 1997).

2.6 Sec31p

Characterization of the 700-kDa Sec13p complex revealed that it is composed of two partners, Sec13p and a protein called "p150" (Salama *et al.*, 1993). Peptides generated from p150 were used to design PCR fragments, which were then used in isolating the *SEC31* gene from a yeast genomic library (Salama *et al.*, 1997).

Sec31p is a hydrophilic, 1273-amino acid protein. It contains 7 WD repeats in the amino-terminal part, and the carboxyl-terminal segment harbors a region rich in serine, threonine, and proline residues (Salama *et al.*, 1997).

The functional unit of the Sec13/31p complex turned out to be a hetero-tetramer, with two molecules of each protein (Lederkremer *et al.*, 2001). The discrepancy between the apparent molecular weights of the complex purified by gel filtration (~700 kDa) and that of the tetramer (calculated to be ~360 kDa) is probably due to the elongated structure of the tetramer (Lederkremer *et al.*, 2001; Matsuoka *et al.*, 2001). This was also suggested by Salama *et al.* (1997), who noted that when the Sec13/31p complex was purified by sedimentation through glycerol gradients, it behaved like a 166-kDa protein.

Apparently, Sec31p interacts with Sec13p *via* its WD repeat domain, *i.e.* the N-terminus (Shaywitz *et al.*, 1997),

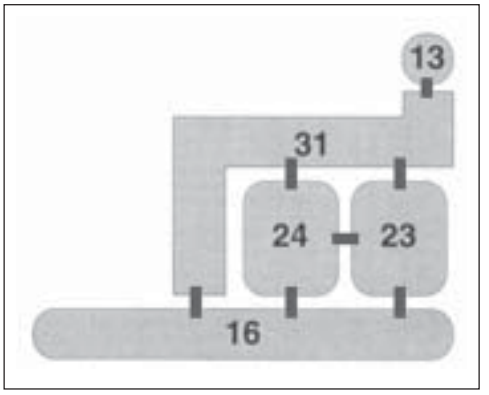


Figure 3. Proposed direct interactions between the different COPII coat proteins. Picture modified from Shaywitz *et al.* (1997).

although indirect evidence suggests that the C-terminal domain of Sec31p is also important for Sec13p binding (Salama *et al.*, 1997). However, based on yeast two-hybrid assays and binding studies of purified proteins in solution, Sec31p lacking the N-terminus is unable to bind Sec13p. Moreover, the same binding studies demonstrated that the C-terminal portion of Sec31p interacted directly with Sec16p, a peripheral membrane protein at the ER membrane (see Section 2.7), but not with Sec13p (Shaywitz *et al.*, 1997). In addition, Sec31p appears to interact directly also with the two other COPII components, Sec23p and Sec24p (Shaywitz *et al.*, 1997). This is likely to stabilize the coat during assembly (Section 3.4). Postulated interactions between Sec31p and other COPII proteins are shown in Figure 3.

Sec31p is the only COPII component that is posttranslationally modified by phosphorylation. This modification appears to be important for the function of the protein in vesicle formation since phosphatase treatment significantly reduced the protein's ability to promote vesicle budding in a cell-free assay (Salama *et al.*, 1997).

SEC31/WEB1 was also isolated independently in a screen for yeast mutants that was dependent on expression of the adenoviral oncogene E1A (Zieler *et al.*, 1995). The relevance of this finding is unclear.

2.7 Sec16p

Two mutant alleles of *SEC16* were isolated in the first large screen for secretory mutants (Novick *et al.*, 1980). The gene was cloned from a yeast genomic library by complementation of temperature-sensitive growth of a *sec16-1* mutant. Sec16p is a 2194-amino acid/240-kDa peripheral membrane protein associated with the ER and ER-derived vesicles (Espenshade *et al.*, 1995).

Several studies indicate a role for Sec16p in vesicle budding. The *SEC16* gene shows interactions with several genes required for vesicle formation. The *sec16-1* mutation is synthetically lethal (*i.e.* double mutants are inviable) in combination with *sec12-1*, *sec13-1*, and *sec23-1* (Kaiser and Schekman, 1990). Furthermore, the temperature-sensitive growth defect of *sec16-2* can be suppressed by overproduction of Sar1p (Nakano and Muramatsu, 1989), the Sec23/24p complex (Kurihara *et al.*, 2000), or Sed4p (Section 2.8; Gimeno *et al.*, 1995). In addition, overproduction of N-terminally truncated Sec16p suppresses growth defect in a temperature-sensitive *sar1* background (Saito *et al.*, 1999). These genetic analyses are supported by results from yeast two-hybrid assays and binding studies performed with purified protein fragments (Shaywitz *et al.*, 1997). They demonstrate that Sec16p interacts directly with Sec23p, Sec24p, and Sec31p (see Figure 3).

2.8 Sed4p

Sed4p is a 1065-amino acid type II integral membrane protein located at the ER membrane. The cytosolic domain of the protein shares significant homology with Sec12p, the GEF for Sar1p (Hardwick *et al.*, 1992).

Support for a role in ER-to-Golgi traffic was obtained when overexpression of Sed4p was discovered to suppress the

temperature-sensitive growth of *sec16-2*. Furthermore, the interaction between the two proteins appears to be direct (Gimeno *et al.*, 1995). In addition, deletion of *SED4* lowers the restrictive temperatures of strains that have mutations in genes affecting vesicle formation (Gimeno *et al.*, 1995). Like Sec12p, Sed4p appears to recycle between ER and Golgi in a Rer1p-dependent way (Sato *et al.*, 2001), although it cannot be detected in COPII vesicles (Gimeno *et al.*, 1995).

3. Sequence of events: formation of vesicles at the ER membrane

3.1 Overview

Assembly of the COPII coat on the ER membrane is initiated by activation of Sar1p by the ER-localized Sec12p, the GEF for Sar1p (Barlowe and Schekman, 1993). The activated Sar1p-GTP attaches to the ER membrane (d'Enfert *et al.*, 1991b), and then the membrane-associated Sar1p-GTP recruits the Sec23/24p heterodimer to the membrane. Sar1p-GTP and Sec23/24p form a prebudding complex that can interact with cargo proteins (Kuehn *et al.*, 1998; Springer *et al.*, 1999). Finally, binding of the Sec13/31p tetramer to the prebudding complex completes the coat, and the COPII-coated vesicle pinches off the membrane (Matsuoka *et al.*, 1998). Figure 4 shows the step-by-step assembly of the COPII coat.

A method based on light scattering has recently been developed to allow real-time "visualization" of coat assembly on synthetic liposomes (Antonny *et al.*, 2001). As these defined reactions lack all of the players that are present *in vivo*, the conclusions that can be drawn from the experiments are limited. Despite this, these real-time studies are a valuable aid in understanding the budding event.

3.2 Initiation: recruitment of Sar1p GTPase to the ER membrane

3.2.1 Activation of Sar1p by Sec12p

The cytosolic domain of Sec12p, an integral ER membrane protein, stimulates GDP/GTP exchange on Sar1p (Barlowe and Schekman, 1993). As a result, Sar1p-GTP associates with the ER membrane (Figure 4). The location of Sar1p at the membrane marks the site where a new vesicle begins to grow.

3.2.2 Membrane attachment of Sar1p

How is the Sar1 protein attached to the membrane? Sar1 proteins are most closely related to ARF proteins but, unlike these, do not have any potential sites for posttranslational lipid modifications that could explain the membrane-binding affinity of the protein (Oka *et al.*, 1991). Fortunately, crystallographic analyses of both mammalian Sar1* (Huang *et al.*, 2001) and the yeast prebudding complex (see Section 3.3.1), composed of Sar1p and the Sec23/24p complex (Bi *et al.*, 2002), have now been resolved. This has enabled

* The mammalian COPII proteins are written without "p". It is an established practice that helps to distinguish between yeast and mammalian COPII components.

formulation of hypotheses based on more than mere guesswork.

Two alternative models have been proposed. First, Huang *et al.* (2001) determined the three-dimensional structure of hamster Sar1 in its inactive, GDP-bound form. Based on the structure obtained they were able to design mutant forms of Sar1 in a directed fashion. The Sar1 variants were then tested for the ability to promote vesicle formation in a cell-free assay and for the ability to bind the Sec23/24 complex.

The first nine amino acids from the N-terminus of Sar1 form a motif that is unique for the Sar1 family. This motif was designated STAR (from Sar1-NH_2-terminal activation recruitment), and it contains several bulky, hydrophobic amino acids such as phenylalanine and tryptophan (Huang *et al.*, 2001). A single point mutation [F5D] in this area rendered the protein unable to bind to membranes in the presence of either GTP or GTPγS, although it did not affect the nucleotide-binding properties of the protein. The authors concluded that the hydrophobic STAR motif mediates the attachment of Sar1 proteins to the ER membrane *via* interactions with the lipid bilayer and perhaps also interacts directly with membrane-located Sec12 protein (Huang *et al.*, 2001).

Second, Bi *et al.* (2002) presented a model for membrane attachment for Sar1p based on the structure of the yeast COPII prebudding complex. The Sar1p portion was crystallized with a nonhydrolyzable GTP analog, GppNHp.

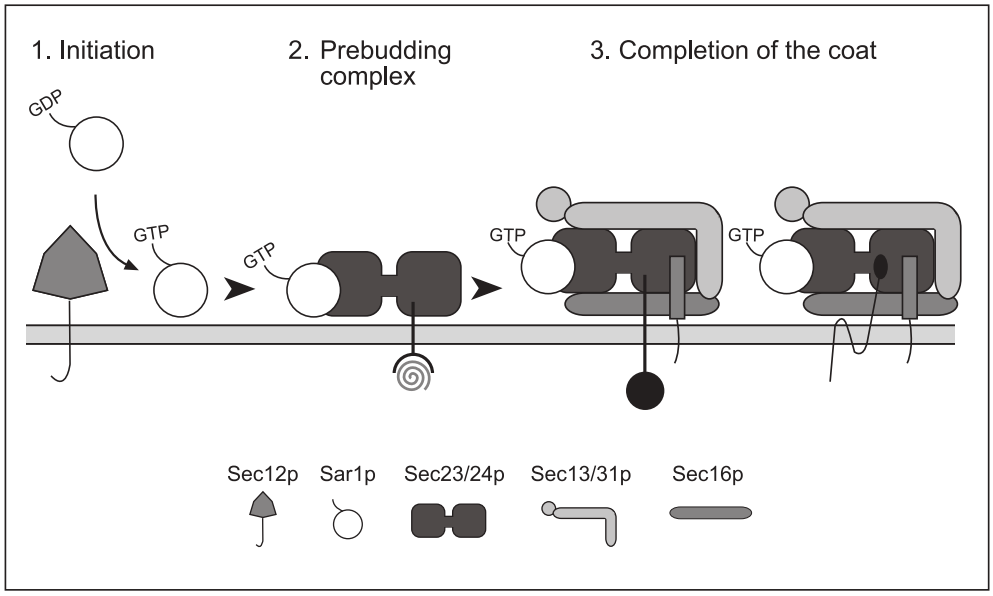


Figure 4. Step-wise assembly of the COPII coat. 1. Activation of Sar1p by Sec12p at the ER membrane initiates coat assembly. 2. Activated Sar1p attaches to the ER membrane and recruits Sec23/24p; the three proteins form the prebudding complex, which is able to interact with cargo proteins. 3. Recruitment of Sec13/31p completes the coat. The peripheral membrane protein Sec16p is a member of the COPII coat, and interacts with Sec23p, Sec24p, and Sec31p.

Thus, the structure obtained represented Sar1p in its active form.

With ARF proteins, the myristoyl tail in the N-terminus is exposed in ARF-GDP and shows low affinity towards membranes in the GDP-bound form (Franco *et al.*, 1995). However, the membrane attachment of ARF-GTP occurs *via* an amphipathic α-helix close to the N-terminus, not the myristoyl tail (Franco *et al.*, 1993, 1996; Antonny *et al.*, 1997).

By comparing the crystal structures of Sar1 proteins in inactive (Huang *et al.*, 2001) and active forms, Bi *et al.* (2002) proposed that membrane binding of Sar1 and ARF proteins is, in fact, analogous. The extreme N-terminus of Sar1p, the STAR motif, would correspond to the myristoyl tail of ARF, and like ARF proteins, Sar1 proteins would bind to the lipid bilayer *via* the amphipathic α-helix. The authors nicely show that a conformational change due to GTP binding results in the exposure of the amphipathic α-helix, allowing interaction with membranes (Bi *et al.*, 2002).

The conclusions of the two groups are somewhat controversial. If the STAR motif is required just to increase the membrane association of inactive Sar1p to confirm that Sar1p and Sec12p occasionally interact, the important feature would be expected to be the hydrophobicity of the motif. Therefore, it is not easily explained why single amino acid substitutions within the STAR sequence have different outcomes on membrane attachment of Sar1 (Huang *et al.*, 2001). However, the model proposed by Bi *et al.* (2002) is more tempting, as it would mean that the small GTPases are not so divergent after all.

3.2.3 Requirement for trinucleotides in vesicle budding

Guanosine triphosphate, GTP

The yeast *in vitro* studies have shown that generation of vesicles from the ER membrane requires only GTP bound to Sar1p (Barlowe *et al.*, 1994). In addition, GTP can be replaced by its non-hydrolyzable analogs, GMP-PNP or GTPγS (less efficient), demonstrating that formation of vesicles does not require hydrolysis of GTP. However, if one of these analogs is used, much higher concentrations of the Sec23/24p and Sec13/31p complexes are required (Barlowe *et al.*, 1994). Apparently, the amount of the two protein complexes in cytosol preparations is insufficient to drive vesicle formation in the presence of nonhydrolyszable GTP; instead it requires the use of purified COPII components. Earlier *in vitro* studies conducted with GTPγS showed that it inhibits vesicle formation, and GTP hydrolysis was concluded to be important in the budding step (Oka *et al.*, 1991; Rexach and Schekman, 1991; Barlowe *et al.*, 1993). These observations can now be explained to result from the use of cytosol as a source for the COPII proteins.

Hydrolysis of GTP is, however, important in ER-to-Golgi transport, but only at later stages. It is required to promote dissociation of the coat, a prerequisite for fusion of these vesicles with the *cis*-Golgi membranes (Barlowe *et al.*, 1994). Therefore, COPII coats generated in the presence of GMP-PNP or GTPγS are stable and result in accumulation of coated vesicles in the reactions. This has enabled the bio-

chemical and immunological characterization of COPII vesicles. For instance, immuno-EM of coated vesicles has confirmed that the COPII coat indeed contains Sar1p, Sec13/31p complex, and Sec23/24p complex (Barlowe *et al.*, 1994). In addition, identification of proteins that have been recruited into, or excluded from, the vesicles has been very informative when, for example, characterizing the cargo selection properties of COPII coats (Kurihara *et al.*, 2000; Shimoni *et al.*, 2000).

Adenosine triphosphate, ATP

In yeast cells, ATP is not required for the budding reaction *per se*, as mentioned above. However, if crude cytosol instead of purified proteins is used as a source of COPII components, the *in vitro* reaction becomes dependent on ATP and an ATP regeneration system in the presence of GTP (Rexach and Schekman, 1991). Apparently, phosphatases present in the cytosol preparation hydrolyze the supplemented GTP. The function of ATP is probably to maintain GTP at sufficient levels, as also GDP can in these conditions support vesicle formation (Barlowe *et al.*, 1994).

In mammalian cells, ATP has been suggested to be directly required for COPII vesicle budding from ER membranes (Aridor *et al.*, 1998; Aridor and Balch, 2000). First, *in vitro* reactions in the mammalian system suggest that the purified Sec23/24 complex, or monomeric Sec23 (which also binds to membranes in a Sar1-dependent way in this system), is recruited to the membrane only in the presence of ATP regardless of whether the nucleotide used is GTP or GTPγS (Aridor *et al.*, 1998). Second, the kinase inhibitor H89 was found to abolish membrane binding of the Sec23/24 complex in the presence of ATP and GTPγS (Aridor and Balch, 2000).

Thus, reliable studies appear to exist both for and against the direct role of ATP in COPII recruitment. Both yeast and mammalian systems show a requirement for ATP if the COPII components have to be recruited from the cytosol. However, purified COPII proteins, which can be supplemented in higher concentrations than what is present in cytosol preparations, can form vesicles in the yeast system in the presence of GMP-PNP or GTPγS only (Barlowe *et al.*, 1994). By contrast, GTPγS alone appears to be insufficient to recruit purified monomeric Sec23 or the Sec23/24 complex to the ER membrane (Aridor *et al.*, 1998). In addition, in mammalian systems, the recruitment of Sar1-GTPγS to ER membranes has been shown to be sensitive to the kinase inhibitor H89, suggesting that ATP is directly required in the initiation step of vesicle formation in mammalian cells (Aridor and Balch, 2000). Thus, regulatory elements in COPII vesicle formation may show slight variations between yeast and mammalian cells.

3.3 Recruitment of Sec23/24p: formation of the prebudding complex

After the Sar1p-mediated initiation step, the next cytosolic component to be recruited to the prebudding complex is the Sec23/24p complex. Together, these three proteins form a prebudding complex that is responsible for selecting the passengers of the vesicle (Figure 4).

3.3.1 Structure of the prebudding complex

As mentioned above, the crystal structure of the yeast prebudding complex was recently elucidated (Bi *et al.*, 2002). This study is without doubt going to have an enormous impact on research of vesicle formation and cargo selection, as these events can now be approached from a completely different point of view, the atomic level.

The crystal structure of the prebudding complex reveals that Sec23p and Sec24p fold almost identically, and the shape of the proteins resembles the distal end of a femur. The interface between Sec23p and Sec24p is narrower, giving the overall appearance of a bow tie. Sar1p is more globular and its binding site in the Sec23p molecule is well apart from that of Sec24p (Figure 5). The surface structure of the Sec23/24p complex was previously resolved also at the EM level and likewise shown to resemble a bow tie (Lederkremer *et al.*, 2001; Matsuoka *et al.*, 2001).

Both Sec23p and Sec24p have five distinct domains: a β-barrel, a zinc finger, a "trunk" domain, an all-helical region, and a gelsolin-like domain. Interaction between Sec23p and Sec24p occurs *via* their approximately 250-amino acid trunk domains, whereas three domains (trunk, gelsolin-like, and an all-helical region) of Sec23p participate in binding of Sar1p (Bi *et al.*, 2002).

Inspection of the shape of the prebudding complex has revealed that one side of the Sec23/24p complex is concave. Moreover, the degree of concavity appears to be suitable for covering a 60-nm vesicle, which is approximately the size of an ER-derived vesicle (Lederkremer *et al.*, 2001; Bi *et al.*, 2002). Bi *et al.* (2002) made an important discovery when they noticed that the concave side of the complex has

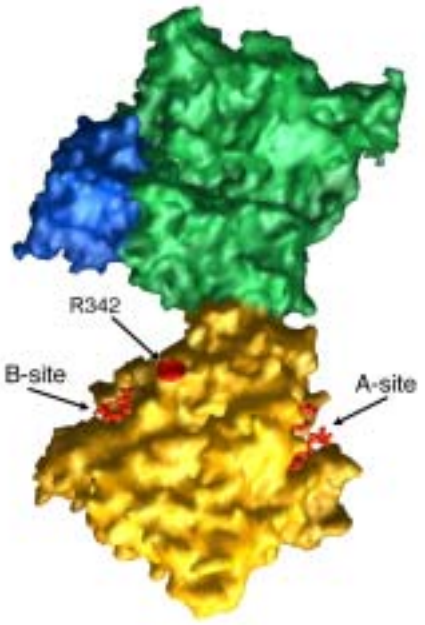


Figure 5. Cargo and SNARE protein binding sites in the prebudding complex composed of Sar1p (blue), Sec23p (green), and Sec24p (yellow). Sed5p and Bet1p binding motifs on Sec24p, A-site and B-site respectively, are indicated in red. R342 of Sec24p is required for Sec22p binding. Another binding motif of Sed5p and the di-acidic ER export signal of Sys1p also interact with the B-site. The surface of the prebudding complex in contact with the membrane faces forward. Surface representation of the prebudding complex was kindly provided by Jonathan Goldberg (Memorial Sloan-Kettering Cancer Center, New York, USA).

a general positive charge. Therefore, it is probable that the concave side of the complex faces the ER membrane, which itself has a net negative charge due to phospholipids. For the first time, these results shed light on the possible role of the COPII proteins in curving the membrane during vesicle formation. Likely, both the shape and the positive charge of the complex are important features in this process (Bi *et al.*, 2002).

3.2.2 Sec23p stimulates GTP hydrolysis on Sar1p

Sar1p in its GTP-bound form at the ER membrane induces the binding of Sec23p in conjunction with Sec24p. Similarly to other GAP proteins, Sec23p binds to the switch I and II regions of Sar1p that surround the nucleotide-binding pocket. These regions in GTPases mediate the energy donation from GTP hydrolysis: two invariant amino acids (threonine in switch I and glycine in switch II; threonine also interacts with Mg^{2+}) interact with the γ-phosphate of GTP. This results in stretching of the switches towards GTP; GEFs are required to accomplish this state. Thereafter, a GAP protein binds to the switch regions and, by supplying an arginine side-chain (arginine finger) to the active site of the GTPase, triggers the hydrolysis of GTP, which, in turn, results in "relaxation" of the switch regions (reviewed in Vetter and Wittinghofer, 2001). Sec23p also possesses the arginine finger (Bi *et al.*, 2002).

Sec23p stimulates GTP hydrolysis on Sar1p by 10- to 15-fold (Yoshihisa *et al.*, 1993). However, the interaction between Sar1p-GTP and Sec23p does not, apparently, result in immediate hydrolysis of GTP. This can be concluded from the real-time assays with purified COPII proteins (Antonny *et al.*, 2001). Addition of Sec23/24p to membranes harboring activated Sar1p-GTP resulted in relatively slow hydrolysis, whereas when both Sec23/24p and Sec13/31p were added, the hydrolysis of GTP on Sar1p was rapid (Antonny *et al.*, 2001). The real-time assay revealed that addition of Sec13p/31p caused a further 10-fold induction in GTP hydrolysis. After hydrolyzing the bound GTP, Sar1p dissociates from the membrane, and thus, the membrane association of Sec23/24p is labile.

As the GAP of Sar1p is one of the members of the coat itself, and moreover, the first to be recruited on the initiated bud, this poses the problem with timing of GTP hydrolysis. Apparently, additional factors must regulate the activity of Sec23p and stabilize the immature coat. If Sar1p hydrolyzes its bound GTP prematurely, it would likely result in disassembly of the prebudding complex.

3.4 Role of Sec16p

Five purified proteins, Sar1p, the Sec23/24p complex and the Sec13/31p complex, are sufficient to stimulate release of COPII-coated vesicles from chemically defined liposomes, when supplemented with nonhydrolyzable GTP analog GMP-PNP (Matsuoka *et al.*, 1998). However, 2.5- to 5-fold higher concentrations of purified COPII components are required when using synthetic liposomes instead of authentic ER membranes. This suggests that the process of vesicle budding in living cells may be more complex. In addition, GMP-PNP, but not GTP, supported vesicle formation in

reactions performed on synthetic liposomes (Matsuoka *et al.*, 1998). Some factor either at the ER membrane or in the cytosol is probably required to prevent the premature hydrolysis of GTP.

An interesting protein in this regard is Sec16p. It shows both genetic and physical interaction with other COPII components (Section 2.7) and has been shown to be a constituent of the COPII vesicles (Espenshade *et al.*, 1995). Despite these strong indications for being a member of the COPII coat, the role of Sec16p has often been ignored. Although temperature-sensitive *sec16* alleles block vesicle formation *in vivo* (Kaiser and Schekman, 1990; Espenshade *et al.*, 1995), this function in *in vitro* reactions was overlooked (Barlowe *et al.*, 1994), probably due to the unusually tight attachment of Sec16p to the membranes (Espenshade *et al.*, 1995). The ER membranes used as a source of vesicles in a typical *in vitro* reaction were usually washed with 2.5 M urea. This treatment was assumed to detach all peripheral membrane proteins from the ER. Thus, only the proteins that were subsequently added to the reaction mixture were considered to be affecting the reaction (Barlowe *et al.*, 1994). However, Sec16p can be partially solubilized with 0.5 M NaCl or high pH, but not with 2.5 M urea (Espenshade *et al.*, 1995). Therefore, the requirement of Sec16p for vesicle budding from the yeast ER membranes was satisfied by the donor membranes themselves.

Although it is now quite evident that Sec16p is part of the COPII coat, interpretation of its role has not been very easy. For example, *in vitro* budding studies performed with synthetic liposomes instead of yeast membranes clearly showed that vesicles can be generated without supplying Sec16p (Matsuoka *et al.*, 1998).

However, a working model for the function of Sec16p now exists. Supek *et al.* (2002) recently suggested that Sec16p and Sar1p-GTP may interact directly. They showed that Sec16p was recruited to defined liposomes in a Sar1p-GTP-dependent manner. The authors suggest that after interacting with Sec12p at the ER membrane, Sar1p binds to Sec16p. This way Sec16p is correctly positioned at the vesicle initiation site and is able to interact with the Sec23/24p complex and with Sec31p of the Sec13/31p complex (see Figures 3 and 4). When Sar1p hydrolyzes its GTP and dissociates from the coat, Sec16p (together with Sec31p) would stabilize the coat structure so that the coat is not shed before the vesicle has pinched off the membrane (Supek *et al.*, 2002).

3.5 Role of Sed4p

The function of Sed4p has remained unclear. However, genetic interactions indicate that Sed4p might function in ER-to-Golgi transport at the vesicle-budding step. First, *SED4* is a multicopy suppressor of temperature-sensitive *sec16* mutations. Second, deletion of *SED4* exacerbates growth defects of temperature-sensitive COPII mutants (Gimeno *et al.*, 1995). Furthermore, deletion of *SED4* from wild-type cells slightly retards transport of vacuolar CPY from ER to the Golgi, and the cytosolic domain of Sed4p interacts directly with the C-terminal part of Sec16p at the ER membrane (Gimeno *et al.*, 1995).

The cytosolic domain of Sed4p is highly related to that of Sec12p (Hardwick *et al.*, 1992). However, attempts to detect GEF activity of Sed4p on Sar1p have failed (Gimeno *et al.*, 1995; Saito-Nakano and Nakano, 2000). This suggests that either Sed4p has no GDP exchange activity or that an as yet unidentified small GTPase is the target protein for Sed4p.

Saito-Nakano and Saito (2000) noted that overproduction of Sed4p lowered the restrictive temperature of temperature-sensitive *sec23* mutations. They also detected slight inhibition of GTPase activity of Sar1p *in vitro* when the cytosolic domain of Sed4p was added to the mixture of Sar1p-GTP and Sec23/24p. Based on this, they suggested that Sed4p might function by slightly inhibiting the GAP activity of Sec23p. If this is the case, then one could speculate that Sed4p is required to decrease the GAP activity of Sec23p towards Sar1p-GTP before the Sec23/24p complex is firmly connected to Sec16p. The binding sites for Sed4p and Sec23p are both in the C-terminal segment of Sec16p, although their precise positions are unknown. When the prebudding complex has been stabilized *via* interactions with Sec16p, Sed4p could dissociate from the complex. However, this is only one of several possibilities. Further evidence is required to clarify the role of Sed4p.

3.6 Cargo is selected at the prebudding stage

Disrupting the function of the COPII coat by incubating a temperature-sensitive COPII mutant at restrictive temperatures causes a complete block in ER-to-Golgi traffic (Novick *et al.*, 1980), indicating that no alternative ER-to-Golgi route exists. Thus, the only way for a secretory protein to travel to the Golgi is to be incorporated into a vesicle covered with COPII proteins. It is today quite evident that most proteins do not travel between organelles by bulk flow. Rather, during vesicle formation, cargo proteins are actively sorted from resident proteins (Schekman and Orci, 1996). The first sorting event is at the ER exit site, where proteins that are to be transported beyond the ER are sorted from resident components. Therefore, it is surprising that thus far only two classes of ER export signals have been identified on the cytosolic tails of some cargo proteins in various organisms. The C-terminus of the p24 family proteins and ERGIC-53/Emp47p possesses a dihydrophobic motif that enhances the ER exit of these proteins (Kappeler *et al.*, 1997; Nakamura *et al.*, 1998; Sato and Nakano, 2002).

The other identified signal is the short diacidic signal of Asp-X-Glu or Glu-X-Glu found in the cytoplasmic tails of at least VSV G (Nishimura and Balch, 1997), some mammalian potassium channels (Ma *et al.*, 2001), and yeast Sys1p (Votsmeier and Gallwitz, 2001).

3.6.1 Sec24p has several binding sites for SNARE proteins

The COPII prebudding complex has an important role in selection of cargo to the forming vesicle. For example, both the dihydrophobic and the diacidic signals have been shown to interact directly with the Sar1-GTP/Sec23/24 prebudding complex (Kappeler *et al.*, 1997; Aridor *et al.*, 1998; Dominguez *et al.*, 1998;

Votsmeier and Gallwitz, 2001; Nufer *et al.*, 2002). A nice demonstration of this is also that the prebudding complex can be recovered in association with cargo proteins when mammalian or yeast microsomes are first incubated with activated Sar1 protein and the Sec23/24p complex (Aridor *et al.*, 1998; Kuehn *et al.*, 1998). Furthermore, yeast vesicle associated soluble N-ethylmaleimide-sensitive factor (NSF) attachement protein receptors (v-SNAREs) Bet1p and Bos1p showed Sar1p-GTP dependent interaction with the prebudding complex (Springer *et al.*, 1999).

Where exactly do these cargo proteins then bind in the prebudding complex? Peng *et al.* (1999) demonstrated that the yeast target (t)-SNARE for ER-derived vesicles, Sed5p, binds specifically to Sec24p, independently of Sec23p or Sar1p (Peng *et al.*, 1999). The role of the Sec24p subunit became more evident when a Sec24p homolog, Sfb3p, was shown to be required for efficient packaging of the plasma membrane ATPase Pma1p (Roberg *et al.*, 1999; Shimoni *et al.*, 2000).

Mapping the binding sites in Sec24p

An important tool in characterizing the putative binding sites in Sec24p became available when the crystal structure of the yeast prebudding complex was determined (Bi *et al.*, 2002). The first detailed results have recently been obtained. Two laboratories have mapped the binding sites for three SNARE proteins, Sed5p, Bet1p, and Sec22p (Miller *et al.*, 2003; Mossessova *et al.*, 2003).

Mossessova *et al*. (2003) first showed that three of four yeast SNAREs (Sed5p, Bet1p, and Sec22p, but not Bos1p) bound to Sec24p and that these interactions were independent of Sar1p or Sec23p. After dissecting the motifs in Sed5p and Bet1p that are important for binding, they designed short peptides corresponding to these motifs and determined the structure of Sec24p bound to each peptide. The authors discovered two distinct binding sites (A and B) in Sec24p. The A-site appeared to be specific for one of the binding motifs determined for Sed5p. The B-site could be occupied by both Bet1p and the other motif of Sed5p (Figure 5). Although the third SNARE, Sec22p, also bound to Sec24p, the binding site for this protein could not be determined.

Miller *et al.* (2003) used another approach. Based on the available crystallographic data (Bi *et al.*, 2002), they designed a directed alanine-scan mutagenesis for Sec24p and screened for mutants that were impaired in binding Bet1p. The alanines causing defects in Bet1p binding were shown to be in a site identical to the A-site of Sec24p discovered by Mossessova *et al.* (2003). In addition to inability to bind Bet1p, the various Sec24p mutants generated also affected binding of two other SNAREs, Bos1p and Sec22p.

One of the alanine-scanning mutants (R342A) was specifically defective in binding Sec22p, although all other proteins were efficiently bound. This R342 is at the surface of Sec24p, but the location of the amino acid is outside of the A- and B-sites. Therefore, it represents a third independent binding site for transmembrane cargo proteins (see Figure 5).

Further characterization of the Sec24p mutants deficient in binding of Bet1p

showed that although they were unable to bind Bet1p, the vesicles generated in a vesicle budding assay contained a variety of other cargo. This indicates that there are additional binding sites for transmembrane cargo in the coat proteins. Furthermore, recent studies suggest that some cargo proteins may also be able to bind Sar1p (Aridor *et al.*, 2001; Belden and Barlowe, 2001a) and even Sec13p (Belden and Barlowe, 2001a).

Miller *et al.* (2003) also analyzed the contents of the vesicles generated *in vitro* with the binding-deficient Sec24p. It appeared that proteins possessing the same ER export signals were differently selected to the vesicles. Sys1p and Gap1p both have a diacidic signal (Votsmeier and Gallwitz, 2001; Malkus *et al.*, 2002). Still, Sys1p was not packaged, whereas Gap1p was. Other ER export signals (dihydrophobic/aromatic) also showed different binding properties. This emphasizes the complexity of cargo selection into a transport vesicle.

Homologs of Sec24p

Sec24p has two functional homologs in yeast, Sfb2p (or Iss1p/Sec24B) and Sfb3p (or Lst1p/Sec24C). Studies performed with the alanine-scanning mutants revealed that both Sec24p homologs were partially able to compensate for the mutated Sec24p *in vivo*. Furthermore, a similar binding site (corresponding to B-site) was mapped in Sfb3p (Miller *et al.*, 2003), suggesting that binding properties, though not specificities, are conserved between homologs. The role of the Sec24p homologs is discussed in more detail in Results and Discussion, Section 1.3.3.

3.7 Completion of the coat

The final stage in coat assembly is the recruitment of the Sec13/31p tetramer (Figure 4; Antonny *et al.*, 2001). This results in polymerization of the coat through interactions of Sec31p with Sec16p, Sec23p, and Sec24p (Shaywitz *et al.*, 1997) as well as in further induction of GTPase activity of Sar1p; binding of the Sec13/31p complex to the membrane results in induction of GTP hydrolysis (Antonny *et al.*, 2001).

According to the information obtained by EM and from the crystal structure of the prebudding complex, the COPII coat is apparently formed from two layers. The crystal structure reveals that the prebudding complex is approximately 4 nm thick (Bi *et al.*, 2002), whereas the whole coat, judged from EM analysis of cross-fractured vesicles covered with COPII, is close to 10 nm (Matsuoka *et al.*, 2001). This suggests that Sar1p and Sec23/24p are constituents of the inner layer and Sec13/31p forms the outer layer (Matsuoka *et al.*, 2001; Bi *et al.*, 2002). Contribution of Sec16p to the thickness of the COPII coat is unclear. The structure of the protein has not been resolved and EM analysis of the coat was performed on vesicles generated from synthetic liposomes with the aid of minimum set of purified proteins: Sar1p, the Sec23/24p complex, and the Sec13/31p complex.

Sec13/31p has been considered a structural component of the COPII coat required for its polymerization (Lederkremer *et al.*, 2001). Recruitment of the Sec13/31p complex also increases the GAP activity of Sec23p, perhaps by optimizing the orientation of Sec23p with respect to Sar1p (Matsuoka *et al.*, 1998).

However, one *in vitro* study suggests that Sec13p might also bind to the cytosolic tails of two yeast proteins, Emp24p and Erv25p, members of the p24 family (Belden and Barlowe, 2001a). The authors suggest that the selection of these cargo proteins into COPII vesicles occurs *via* interactions between the tails of Emp24p and Erv25p with the prebudding complex, and that the interaction with Sec13p is a later event. This finding indicates that cargo proteins may be more than passive passengers in transport vesicles, potentially playing an active role in ensuring successful transportation from the ER to the Golgi.

AIMS OF THE STUDY

Segregation of cargo and resident proteins within the secretory pathway is essential for maintaining organelle identity. The mechanisms governing how various cargo proteins are selected to the transport vesicles have been intensively studied during the last few years. The first decision of sorting takes place in the ER: cargo proteins are selectively packaged into COPII-coated vesicles, whereas resident ER proteins are excluded from entering these vesicles. The components of the COPII coat, especially Sec24p, have been shown to play an important role in this selection process.

The aim of this study was to investigate the mechanisms of cargo selection of soluble secretory proteins *in vivo* using yeast *S. cerevisiae* as a model organism.

First, the selective ER exit of soluble glycoprotein Hsp150 was investigated in conditions where vesicle formation was driven by mutated COPII subunits. The possible role of alternative COPII components in recruitment of Hsp150 into ER-derived vesicles was also examined.

Second, mammalian BAP31 has been suggested to function as a cargo receptor enhancing ER exit of a subset of cargo proteins. Thus, BAP31 homologs in yeast were identified, and whether the function of these homologs is important for enhancing ER exit of various yeast proteins was investigated.

MATERIALS AND METHODS

The yeast strains used in the studies are listed in Table 1, and descriptions of the relevant *sec* mutations are presented in Table 2. Table 3 displays the experimental methods used in studies I-III. The protein constructs used are presented in Figure 6.

Table 1. Yeast strains used in the studies.

Strain	Relevant mutation	Fusion protein	Study	Reference/Source
SEy2101a	none		I, II	R. Schekman
W303-1A	none		I, III	K. Kuchler/J. Thorner
W303-1B	none		I	K. Kuchler/J. Thorner
NY179	none		III	P. Novick
CKY263	none		III	C. Kaiser
SF821-8A	*sec7-1*		I, II	R. Schekman
SF226-1C	*sec12-4*		I	R. Schekman
HMSF163	*sec13-1*		I	Novick *et al.* , 1980
mBy12-6D	*sec18-1*		I, II	R. Schekman
HMSF190	*sec23-1*		I	Novick *et al.* , 1980
RSY282	*sec23-1*		II	R. Schekman
E382-6	*sec24-1*		II	C. Kaiser
JPY206	*sfb2Δ*		II	J. P. Paccaud
JPY207	*sfb3Δ*		II	J. P. Paccaud
JPY205	*sfb2Δ sfb3Δ*		II	J. P. Paccaud
CKY555	*sec31-2*		I	C. Kaiser
RSY153	*sec63-1*		I	R. Schekman
H23	*hsp150::URA3*		I, II	Russo et al., 1992
H340	*sec7-1*	Hsp150Δ-β-lactamase	I	Simonen et al., 1994
H393	*sec18-1*	Hsp150Δ-β-lactamase	I	Simonen et al., 1994
H430	*hsp150::URA3*	Hsp150Δ	I, II	This study
H440	*sec18-1*	Hsp150Δ	I, II	This study
H675	none	Hsp150Δ-β-lactamase	I	Suntio et al., 1999
H839	none	SUI-β-lactamase	I	Suntio et al., 1999
H840	*sec18-1*	SUI-β-lactamase	I	Suntio et al., 1999
H1064	*sec13-1*		I	This study
H1065	*sec13-1*	Hsp150Δ-β-lactamase	I	This study
H1067	*sec13-1*	SUI-β-lactamase	I	This study
H1107	*sec13-1*	Hsp150Δ	I	This study
H1217*§	none		III	Keränen lab collection
H1233	*hsp150Δ::loxP-KanMX-loxP*		I, II	This study
H1234	*hsp150Δ::loxP-KanMX-loxP*		I	This study
H1236	*sec13-1*		I, II	This study
H1237	*sec24-1*		II	This study
H1282*	*yet1Δ::URA3*		III	This study
H1283*	*yet1Δ::URA3*		III	This study
H1284	*sec13-1*		I	This study
H1290*	*yet2Δ::KanMX4^R*		III	This study
H1291*	*yet2Δ::KanMX4^R*		III	This study
H1292*	*yet1Δ::URA3 yet2Δ::KanMX4^R*		III	This study
H1293*	*yet1Δ::URA3 yet2Δ::KanMX4^R*		III	This study
H1400	*sec13-1*	SUI-R3-β-lactamase	I	This study
H1429	*sec13-1*	SUI-Cterm	I, II	This study
H1431	*sec63-1*	SUI-R3-β-lactamase	I	This study
H1432	*sec18-1*	SUI-R3-β-lactamase	I	This study
H1433	*sec7-1*	SUI-R3-β-lactamase	I	This study
H1455	*sec7-1*	Hsp150Δ-HRP	II	This study
H1458	*sec24-1*	Hsp150Δ-HRP	II	This study

Table 1. Continued.

Strain	Relevant mutation	Fusion protein	Study	Reference/Source
H1459	*sec23-1*	Hsp150Δ-HRP	II	This study
H1499	*sec24-1 hsp150Δ::loxP-KanMX-loxP*	Hsp150Δ	II	This study
H1500	*sec24-1*	SUI-Cterm	II	This study
H1508	*hsp150Δ::loxP-KanMX-loxP*	SUI-Cterm	I	This study
H1540	none	SUI-Cterm-invertase	I	This study
H1541	*sec13-1*	SUI-Cterm-invertase	I	This study
H1542	*sec18-1*	SUI-Cterm-invertase	I	This study
H1544	*sec24-1*	SUI-Cterm-invertase	II	This study
H1545	*sec13-1 hsp150Δ::loxP-KanMX-loxP*	Hsp150Δ	I	This study
H1555	*sec24-1 sfb2Δ::loxP-KanMX-loxP*		II	This study
H1575	*sec18-1*	SUI-invertase-Cterm	II	This study
H1577	*hsp150Δ::loxP-KanMX-loxP*	SUI-invertase-Cterm	II	This study
H1578	*sec13-1*	SUI-invertase-Cterm	II	This study
H1579	*sec24-1*	SUI-invertase-Cterm	II	This study
H2612*	*yet3Δ::LEU2*		III	This study
H2614*	*yet3Δ::LEU2*		III	This study
SKY28-1A*	*yet1Δ::URA3 yet3Δ::LEU2*		III	This study
SKY28-12A*	*yet1Δ::URA3 yet3Δ::LEU2*		III	This study
SKY29-11B*	*yet2Δ::KanMX4^R yet3Δ::LEU2*		III	This study
SKY29-1D*	*yet2Δ::KanMX4^R yet3Δ::LEU2*		III	This study
SKY30-11A*	*yet1Δ::URA3 yet2Δ::KanMX4^R yet3Δ::LEU2*		III	This study
SKY30-14A*	*yet1Δ::URA3 yet2Δ::KanMX4^R yet3Δ::LEU2*		III	This study

H-numbers marked with * are yeast strains of Sirkka Keränen's laboratory, VTT; other H-numbers denote yeast strains of Marja Makarow's laboratory, Institute of Biotechnology

§ H1217 is derived from NY179 congenic diploid

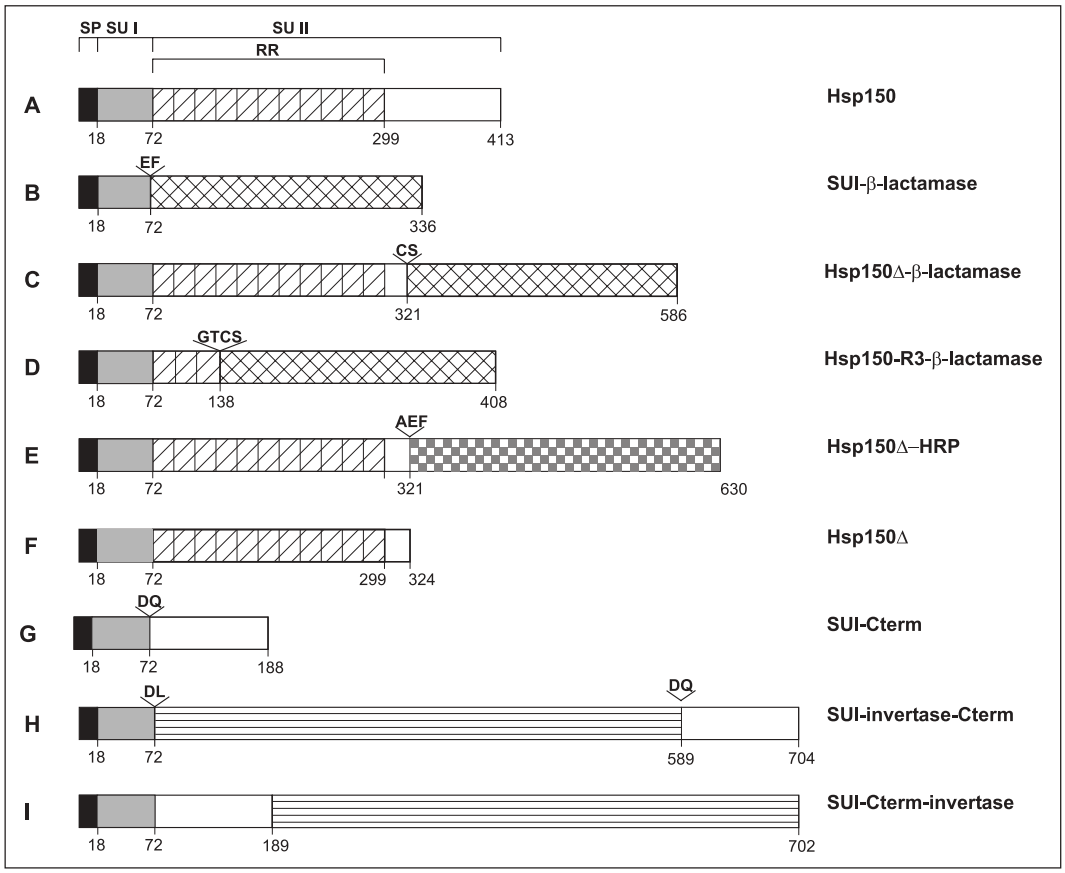
Table 2. Descriptions of relevant yeast mutant phenotypes.

Mutation	Description	Reference
sec7-1	Guanine nucleotide exchange protein (GEF) of Arf-GTPase. Proteins accumulate in the Golgi.	Franzusoff and Scehkman, 1989; Sata *et al.*, 1998; Jones *et al.*, 1999
sec12-4	GEF for Sar1p. Proteins accumulate in the ER.	Nakano *et al.*, 1988; Barlowe and Schekman, 1993
sec13-1	Member of the COPII coat. Proteins accumulate in the ER.	Pryer *et al.*, 1993
sec18-1	Yeast N-metylmaleimide sensitive factor (NSF). Proteins accumulate in the ER and ER derived vesicles.	Kaiser and Schekman, 1990
sec23-1	GAP for Sar1p, member of the COPII coat. Proteins accumulate in the ER.	Hicke and Schekman, 1989; Hicke *et al.*, 1992; Yoshihisa *et al*., 1993
sec24-1	Member of the COPII coat. Proteins accumulate in the ER.	Hicke *et al.*, 1992
sec31-1	Member of the COPII coat. Proteins accumulate in the ER.	Pryer *et al.*, 1993; Wuestehube *et al.*, 1996
sec63-1	Member of the ER translocation machinery required for posttranslational translocation. Proteins accumulate in the cytosol.	Rothblatt *et al.*, 1989

Table 3. Methods used in the studies.

Method	Study	Described in
β-lactamase assay	I	Simonen *et al.* , 1994
Electron microscopy	II	II
Endoglycosidase H digestion	II	II
Generation of antibodies	III	Harlow and Lane, 1988
Immunofluorescence microscopy	I, III	Makarow, 1988; Redding *et al* ., 1991
Immunoprecipitation	I, II	Paunola *et al.* , 1998
Invertase assay	I, III	Makarow, 1988
In vitro translation	III	III
Membrane association assay	III	III
Metabolic labeling of proteins	I-III	Paunola *et al.* , 1998
Nondenaturing PAGE	II	Novick *et al.* , 1980
Northern analysis	III	III
Plasmid construction	I-III	Standard methods
SDS-PAGE	I-III	Laemmli, 1970
Sequencing	III	Sanger *et al.* , 1977
Subcellular fractionation	III	III
Tetrad dissection	III	Standard methods
Western analysis	III	Standard methods
Yeast gene disruption	I-III	Standard methods
Yeast growth assay	III	III
Yeast transformation	I-III	Standard methods

Figure 6. Schematic representations of protein constructs used.



RESULTS AND DISCUSSION

Anterograde transport from the ER to the Golgi complex can be completely inhibited in several ways. Conditional mutations of yeast genes that directly affect this transport step cause accumulation of ER forms of cargo proteins when the cells are incubated in restrictive conditions. These mutants include all genes affecting the formation of COPII vesicles as well as genes required for the subsequent fusion of vesicles with post-ER membranes (Kaiser and Schekman, 1990).

Inhibition of function of the COPI coat, which is required at least for protein transport from the Golgi to the ER in yeast, also results in accumulation of cargo proteins in the ER. Whether the COPI-coated vesicles also transport proteins in an anterograde direction, or perhaps both anterograde and retrograde directions, remains controversial (reviewed in Gaynor *et al.*, 1998). However, it has been confirmed that in yeast the COPI vesicles originate in the Golgi and deliver their cargo to the ER. Selection of proteins into the COPI vesicles is mediated by direct interactions between the cytosolic COPI components and the cytosolic tails of the selected integral membrane proteins (Cosson and Letourneur, 1994). The selected cargo includes factors essential for anterograde traffic, such as SNARE components and putative cargo receptors, both of which need to be continuously cycled between the two compartments. Loss of COPI function is believed to result in depletion of factors important to anterograde traffic from the ER, and consequently, to inhibition of anterograde transport (Gaynor *et al.*, 1998).

Gaynor and Emr (1997) investigated protein secretion in temperature-sensitive COPI mutants at restrictive temperatures and also in conditions where COPI function was abolished by drug treatment. Surprisingly, they noted that anterograde transport is not completely blocked, contrary to earlier assumptions. Two soluble glycoproteins, invertase and Hsp150, were able to bypass the forward transport block caused indirectly by disrupting the COPI pathway (Gaynor and Emr, 1997). The determinants required for directing Hsp150 to the COPI-independent pathway have been identified in our laboratory. They reside in the repetitive region of subunit II (Figure 6A; Suntio *et al.*, 1999).

Figure 6. Hsp150 variants used in the studies. Hsp150 consists of a cleavable signal peptide (SP; black), subunit I (SU I; grey), and subunit II (SU II). Subunit II is divided into a repetitive region (RR; 11 diagonally striped boxes) and a unique C-terminus (white). Chimera proteins were constructed by fusing differentially truncated Hsp150 with β-lactamase (cross-hatched), HRP (checkered), or invertase (horizontally striped). Numbers in constructs indicate the last amino acids in each domain. Letters above constructs indicate amino acids gained in cloning. All variants have a Kex2p cleavage site at the end of subunit I, except for SUI-β-lactamase (B) and Hsp150Δ-HRP (E).

1. Functional COPII components Sec13p and Sec24p are dispensable for ER exit of Hsp150 (I, II)

1.1 Characteristics of Hsp150

HSP150 encodes a protein of 413 amino acids. The *HSP150* promoter confers both constitutive and heat-induced expression of the gene. Incubation at 37°C results in an approximately sevenfold increase in the amount of the Hsp150 protein (Russo *et al.*, 1992).

Hsp150 is a soluble, O-glycosylated protein found both in the culture medium and bound to the cell wall of yeast *S. cerevisiae* (Russo *et al.*, 1992; Kapteyn *et al.*, 1999). It has a cleavable, 18-amino acid signal peptide (Figure 6A) that allows posttranslational translocation of the protein into the lumen of the ER (Paunola *et al.*, 1998, 2001). The signal peptide is followed by subunits I (54 amino acids) and II (341 amino acids). A Kex2p-specific cleavage site between these subunits is processed in the late Golgi. However, the polypeptides remain noncovalently bound to each other in the secreted Hsp150 (Russo *et al.*, 1992).

Subunit II is composed of a repetitive region and a unique C-terminal fragment. The repetitive region consists of 11 repeats of a 19-amino acid peptide with short linkers separating some of the peptides (Russo *et al.*, 1992). The C-terminal fragment has four cysteine residues that form one or two disulfide bonds (Jämsä *et al.*, 1994).

Hsp150 has no N-glycosylation sites, instead it has 21 and 85 potential O-glycosylation sites in subunits I and II, respectively. Based on MALDI-TOF mass spectrometry analyses, secreted subunit I is decorated with 8-50 mannoses and subunit II, on average, with 65 mannose residues (Suntio *et al.*, 1999). Length of the O-glycans in subunit I has not been determined; in subunit II, approximately 60% of the O-glycans are dimannosides. The remaining 40% is comprised of tri-, tetra-, and penta-mannosides in roughly equal amounts (Jämsä *et al.*, 1995).

1.2 Transport of Hsp150 in various COPII mutants

As Hsp150 is transported from the ER to the Golgi in a COPI-independent manner (Gaynor and Emr, 1997), we considered it worth studying ER exit of Hsp150 in COPII vesicles in more detail. However, we thought it unlikely that a COPII-independent route exists given that COPII mutations cause a complete block in ER-to-Golgi transport (Kaiser and Schekman, 1990).

Because the different biosynthetic intermediates of Hsp150 are well documented (Figure 7; Russo *et al.*, 1992; Jämsä *et al.*, 1994; Suntio *et al.*, 1999), we chose pulse-labeling analyses to follow the fate of Hsp150 in various temperature-sensitive COPII mutants. *sec12-4*, *sec13-1*, *sec23-1*, *sec24-1*, and *sec31-2* cells were grown at an early logarithmic phase at a permissive temperature (24°C) and were then shifted to a restrictive temperature (37°C) in a fresh medium. The cells were preincubated for 15 minutes to impose the mutant phenotype and to upregulate the production of Hsp150 from its heat-inducible promoter. After preincubation, the cells were given a 5-minute pulse of

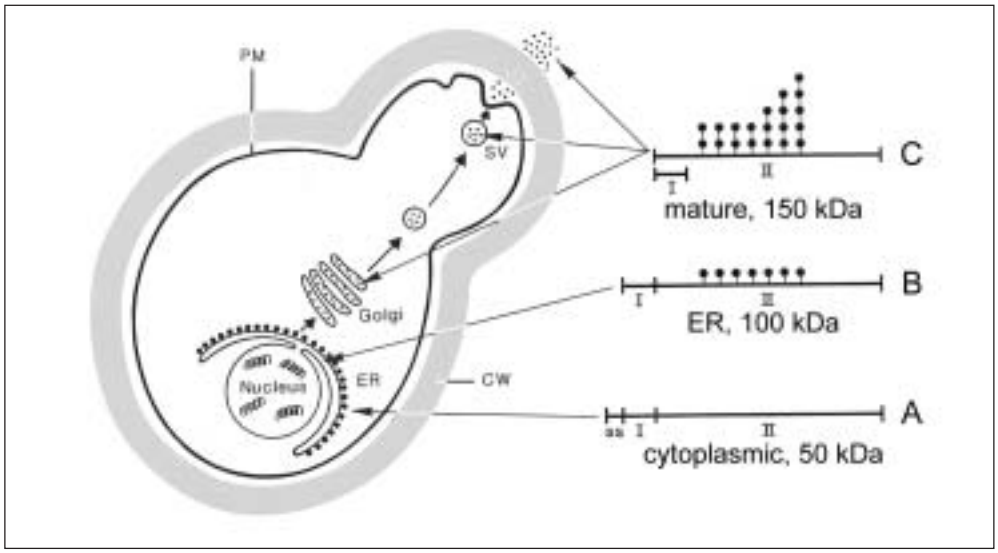


Figure 7. Presentation of the biosynthetic intermediates of Hsp150. (A) Hsp150 is first translated in the cytosol. (B) The signal peptide (SS) of Hsp150 is cleaved when the protein enters the lumen of the ER. The first mannose residue of the numerous O-glycans of the protein is added in the ER. (C) One to four mannoses are sequentially added to O-glycans when the protein is transported through the Golgi. In the *trans*-Golgi, subunit I is cleaved by Kex2p, but it remains noncovalently bound to the rest of the protein. Apparent molecular weights of the protein intermediates are indicated.

^{35}S-methionine/cysteine. Labeled Hsp150 was chased for various times in the presence of cycloheximide (CHX). As expected, in *sec23-1* and *sec31-2* (I, Figure 3), as well as in *sec12-4* (I, data not shown) mutants, the protein remained in the ER.

To our surprise, however, in conditions where ER exit of cargo proteins was inhibited either by a nonfunctional Sec13p or Sec24p, Hsp150 was secreted into the culture medium (I, Figure 1A; II, Figure 2A). In both mutants, secretion of Hsp150 was, however, slower than in wild-type cells, in which Hsp150 is found in the culture medium in less than 5 minutes (Jämsä *et al.*, 1994).

In *sec13-1* and *sec24-1* mutants, a population of mature Hsp150 molecules was detected in the cell lysate. This raised the question of whether the defective COPII proteins participate in the later steps of transport as well. However, the majority of these proteins resided in the cell wall, indicating that this portion was in fact externalized (I, data not shown). Thus, the rate-limiting step in secretion of Hsp150 was exit from the ER.

Although secretion of Hsp150 was slow, it was quite efficient. In *sec13-1* cells, more than 50% of Hsp150 was in the medium after 15 minutes of chase and 82% after one hour (I, Figure 1B). Similar secretion kinetics for Hsp150 was observed regardless of whether the preincubation before labeling was for 15

or 60 minutes (I, Figure 1B); the same applied to *sec24-1* cells preincubated for 15 or 30 minutes (II, Figure 2A).

A portion of the Hsp150 was always found in an immature form inside the mutant cells (I, Figure 1A; II, Figure 2A). It migrated increasingly slowly with prolonged incubation times at 37°C. A population of Hsp150 molecules appeared to be trapped in the ER for an unknown reason. The increase in apparent molecular weight is most likely due to the elongation of the O-glycans of the protein. A recent study showed that two Golgi-localized mannosyltransferase complexes recycle between ER and Golgi (Todorow *et al.*, 2000). These enzyme complexes are responsible for extending the N-glycans of secretory proteins with linear α1,6-linked mannose chains. Our laboratory has thereafter confirmed that, in addition to these mannosyltransferase complexes, Och1p, a mannosyltransferase acting before the two complexes, also recycles (Karhinen and Makarow, 2004). Moreover, this study demonstrated that these Golgi enzymes are enzymatically active in the ER. In wild-type cells, their activity cannot be visualized *in vivo*. However, when formation of ER-derived vesicles is inhibited, the transferases together with their substrates become trapped in the ER. Under these circumstances, the N- and O-glycans of cargo proteins are modified by mannosyltransferases normally located in the early Golgi compartments (Karhinen and Makarow, 2004).

To confirm that the ER-to-Golgi transport of other proteins was arrested in the same cells that continued to secrete Hsp150, the fate of two other cargo proteins was followed. Vacuolar carboxypeptidase Y (CPY) has four glycosylation sites that acquire core oligosaccharides in the ER (p1 form). These glycans are further modified in the Golgi (p2 form), and upon arrival in the vacuole, the N-terminal pro-region is cleaved, generating the mature (m) form (Stevens *et al.*, 1982). Invertase is a soluble, sucrose-hydrolyzing enzyme secreted into the periplasmic space (Carlson and Botstein, 1982). It has nine sites for N-linked glycosylation. Mature invertase migrates in a gel as a smear due to heterogeneously elongated N-glycans (Lehle *et al.*, 1979; Esmon *et al.*, 1981). Synthesis of invertase is derepressed when the amount of glucose is low. ER forms of both CPY and invertase accumulated when *sec13-1* or *sec24-1* cells were incubated at a restrictive temperature (I, Figure 2; II, Figure 2B-C), demonstrating that (1) ER-to-Golgi traffic was truly blocked, and (2) transport of CPY and invertase was dependent on functional Sec13p and Sec24p. This also shows that invertase, which is secreted independently of COPI function (Gaynor and Emr, 1997), cannot bypass the block caused by temperature-sensitive COPII mutants.

From these data, it can be concluded that Hsp150 was slowly but efficiently secreted into the culture medium when COPII component Sec13p or Sec24p was nonfunctional. In the same cells, transport of CPY and invertase was arrested in the ER. Since other components affecting COPII vesicle formation, *i.e.* Sec12p, Sec23p, and Sec31p, were required for transport of Hsp150, the COPII machinery was shown not to be completely dispensable.

Based on the present knowledge, it appears that, in addition to Hsp150, at least a few other proteins have to be selected to this alternative route. These

are the SNARE proteins, C-terminally anchored integral membrane proteins present both in the vesicles and at the target membranes (v- and t-SNAREs, respectively). SNARE proteins are required for homotypic and heterotypic fusion between two membranes; vesicles lacking SNAREs cannot fuse with other membranes (reviewed in Jahn *et al.*, 2003; Burri and Lithgow, 2004). Thus, since Hsp150 is correctly glycosylated and processed by Kex2p before secretion, the SNARE proteins most likely are present in the vesicles containing Hsp150.

SNARE proteins participating in the fusion of ER-derived vesicles in yeast are Bos1p, Bet1p, Sec22p, and Sed5p (Newman *et al.*, 1990; Hardwick and Pelham, 1992; Lian and Ferro-Novick, 1993). Bet1p is a v-SNARE, whereas Bos1p, Sec22p, and Sed5p appear to be functional at the target membrane (Parlati *et al.*, 2000). Of these, Sec22p is the only nonessential SNARE (Dascher *et al.*, 1991), and it can be substituted by another v-SNARE protein, Ykt6p (Liu and Barlowe, 2002).

1.3 Possible explanations for ER exit of Hsp150 in the absence of functional Sec13p or Sec24p

The *sec13-1* mutation is a S224K point mutation in the fifth "blade" of the hypothetical six-bladed propeller structure of Sec13p (see Introduction, Section 2.5; Pryer *et al.*, 1993). How severe the consequences at elevated temperatures that this mutation imposes on the conformation of Sec13p, and consequently, on the capability of the protein to interact with Sec31p, are unknown. In addition, assumptions about the possible participation of the nonfunctional Sec24p in coat assembly cannot be made because the *sec24-1* mutation has not been mapped.

One can speculate that the mutated protein (Sec13p or Sec24p) is still, though poorly, incorporated into the coat. The amount of vesicles formed and/or their cargo selection properties are insufficient to promote ER exit of such proteins as CPY and invertase, while transport of Hsp150, and presumably of SNARE proteins, is allowed.

1.3.1 Coat formation in the absence of Sec13p

Could Hsp150 be transported in coats that completely lack Sec13p? This possibility initially seems quite far-fetched because of the wealth of information about the importance of the COPII proteins for vesicle formation both *in vivo* (*e.g.* Kaiser and Schekman, 1990) and *in vitro* (*e.g.* Barlowe *et al.*, 1994; Matsuoka *et al.*, 1998). It was therefore somewhat surprising that *SEC13* could in fact be completely deleted without affecting the viability of the cell (Elrod-Erickson and Kaiser, 1996). However, this is possible only in cells that have additional mutations. In a screen for suppressors of deletion of *SEC13*, three genes were identified that, when nonfunctional, could complement the lethal phenotype caused by the absence of Sec13p. Although the sorting between cargo proteins and resident ER proteins is slightly impaired in the *bst* mutants (null mutations of *BST1*, *BST2*/*EMP24*, or *BST3*), proteins such as CPY and invertase are transported and correctly modified in these mutants even in the

absence of Sec13p (Elrod-Erickson and Kaiser, 1996). Since the *bst* mutations also slightly suppressed other COPII mutations, the *BST* genes were speculated to be negative regulators of ER exit by preventing incorrectly coated COPII vesicles from pinching off the membrane. In *bst* mutants, the control of coat formation was hypothesized be less stringent, enabling transport to continue in incompletely coated vesicles (Elrod-Erickson and Kaiser, 1996). Unfortunately, the effect of *bst* mutations in a *sec24-1* background was not studied.

Because CPY and invertase were not transported in the *sec13-1* mutant in our study (I, Figure 2), it is highly unlikely that a spontaneous *bst* mutation would have occurred in this strain. Thus, Hsp150 was secreted at a restrictive temperature in a *sec13-1* strain, in which *BST* genes were fully functional. Even if *bst* mutations were able to partially suppress the *sec24-1* phenotype, this was not the reason for secretion of Hsp150 in the *sec24-1* strain used in our study since CPY and invertase were trapped in the ER, indicating that *BST* genes were intact.

1.3.2 Putative Sec13p homologs

Sec13p is composed entirely of WD40 repeats, a common structural domain found in various proteins with diverse functions (Pryer *et al.*, 1993; Garcia-Higuera *et al.*, 1998; Li and Roberts, 2001). The potential candidates for functional Sec13p homologs are therefore difficult to identify. Three putative Sec13p homologs, Seh1p (349 amino acids; 26% identity with Sec13p over 220 amino acids), Lst8p (303 amino acids; 20% identity over 260 amino acids), and Tup1p (713 amino acids; 28% identity over 273 amino acids), have been characterized, but they do not seem to have a direct role in vesicular transport. Seh1p (and Sec13p) has an unknown function in nuclear pore complexes, (see Introduction, Section 2.5.1; Siniossoglou *et al.*, 1996), Lst8p is a negative regulator of biosynthesis of amino acids and is located peripherally at the Golgi membranes (Chen and Kaiser, 2003), whereas Tup1p acts as a general repressor of RNA polymerase II transcription in the nucleus (Keleher *et al.*, 1992). To date, no studies showing interactions between these Sec13p-homologs and Sec31p have been published, and no indications exist that a homologous protein could replace the nonfunctional Sec13p. Therefore, COPII coats in *sec13-1* cells likely either lack the nonfunctional Sec13p or the mutated protein participates in coat assembly poorly (Figure 8A).

1.3.3 Role of Sec24p homologs

The yeast genome harbors two genes, *SFB2* (*ISS1*/*SEC24B*) and *SFB3* (*LST1*/*SEC24C*), which code for functional homologs of Sec24p (Roberg *et al.*, 1999; Kurihara *et al.*, 2000; Shimoni *et al.*, 2000).

Sfb2p (Iss1p/Sec24B)

Sfb2p has a 62% identical amino acid sequence to Sec24p (Kurihara *et al.*, 2000). Elevated levels of Sfb2p suppress the growth defects of temperature-sensitive *sec24* mutations (Higashio *et al.*, 2000; Kurihara *et al.*, 2000; Peng *et al.*, 2000) and also the entire deletion of *SEC24* (Higashio *et al.*, 2000; Kurihara *et al.*, 2000). Similarly to Sec24p, also

Sfb2p can form a complex with Sec23p (Kurihara *et al.*, 2000). As Sfb2p can functionally substitute Sec24p in the COPII coat, it indicates that the cargo selection properties of Sfb2p are sufficiently similar to that of Sec24p for the cell to survive without the function of Sec24p. Indeed, analyses of the contents of the vesicles produced with Sfb2p *in vitro* showed that cargo proteins appeared to be sorted and incorporated correctly: SNARE proteins Sec22p and Bet1p, as well as several cargo proteins, were selected to the vesicles, whereas Sec61p, a resident ER protein, was excluded (Kurihara *et al.*, 2000). In addition, it has been shown that Sfb2p, similarly to Sec24p, can directly bind Sed5p, the t-SNARE component required for fusion of ER-derived vesicles (Peng *et al.*, 1999). However, there might be subtle differences in the properties of Sfb2p and Sec24p that become limiting in certain genetic backgrounds. This is demonstrated by the finding that *SFB2* expressed from a multicopy plasmid cannot always suppress the complete deletion of *SEC24* (Peng *et al.*, 2000).

Thus, it can be hypothesized that when the function of Sec24p is compromised Sfb2p in the place of Sec24p drives vesicle formation and cargo selection (Figure 8B). Whether the mutated Sec24p is also present in the coat, cannot be concluded from the existing data.

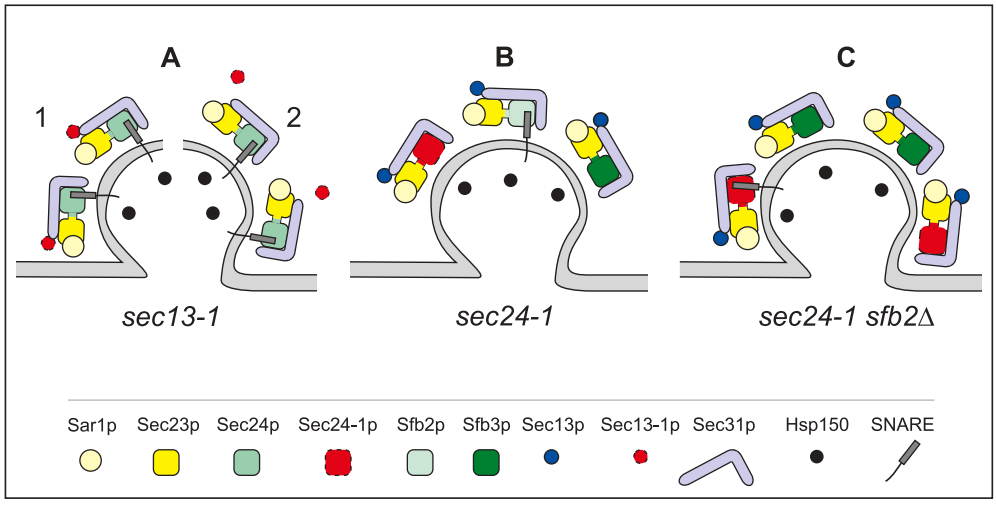


Figure 8. Model for formation of COPII vesicles in the absence of functional Sec13p or Sec24p. (A) In *sec13-1* cells at restrictive temperatures Hsp150 and apparently SNARE proteins are selected to vesicles coated with (1) COPII coats where the nonfunctional Sec13p (Sec13-1p) is poorly attached, or (2) COPII coats lacking Sec13p altogether. (B) In the *sec24-1* mutant, the nonfunctional Sec24-1p coat protein can be substituted by Sfb2p. Whether the mutated Sec24-1p or Sfb3p is present in these vesicles is not known. SNAREs can be selected by Sfb2p. (C) Sfb2p is not required for transport of Hsp150 in a *sec24-1* background at 37°C. Therefore, vesicle formation is possibly driven by mutated Sec24-1p (for selection of SNAREs) or perhaps Sfb3p. The mechanism of recruitment of Hsp150 is unclear.

Sfb3p (Lst1p/Sec24C)

The other Sec24p homolog, Sfb3p, shares 23% identical amino acids with Sec24p (Roberg *et al.*, 1999). Sfb3p has been shown to have a special role; it is required for efficient packaging of Pma1p into COPII vesicles (Roberg *et al.*, 1999; Shimoni *et al.*, 2000). Like Sfb2p, Sfb3p forms a heterodimer with Sec23p and is capable of driving vesicle formation from ER membranes in the absence of Sec24p *in vitro* (Shimoni *et al.*, 2000; Miller *et al.*, 2002). However, overexpression of Sfb3p cannot complement the growth defect of temperature-sensitive *sec24* mutants (Peng *et al.*, 2000). This suggests that the cargo selection properties of Sfb3p are different from Sec24p (and Sfb2p). Indeed, vesicles generated with Sfb3p, although they contain a variety of cargo molecules, lack essential SNARE proteins Bet1p, Bos1p, and Sec22p, which makes the fusion of the vesicle with the target membrane impossible (Miller *et al.*, 2002). This also gives a likely explanation for the inability to compensate for the nonfunctional Sec24p. *In vivo*, the vesicles transporting Pma1p incorporated by Sfb3p also contain Sec24p, which recruits SNAREs required for subsequent fusion with post-ER membranes (Shimoni *et al.*, 2000). A recent study has revealed a binding site in Sfb3p for a subset of cargo proteins. The localization of this site is identical to that of the Bet1p-binding site (B-site) in Sec24p (see Introduction, Section 3.6.1 and Figure 5). Moreover, the amino acids of this site are mostly conserved. However, slight variations in amino acid residues between Sec24p and Sfb3p exist, and that apparently results in different cargo selection properties for the two COPII components (Figure 8B).

1.3.4 Role of Sec24p homologs in ER exit of Hsp150

To study the role of Sec24p homologs in more detail, the ER exit of Hsp150 in strains from which one or both homologs were disrupted was investigated. Single disruptions of *SFB2* or *SFB3* genes, or double disruption *sfb2Δ sfb3Δ* in a *SEC24* background did not alter the transport efficiency or kinetics of Hsp150, indicating that they were not required when Sec24p itself was functional (II, data not shown).

Attempts to disrupt *SFB3* from yeast strains with temperature-sensitive *sec24* mutations have been made, but apparently this combination is lethal (Roberg *et al.*, 1999; Peng *et al.*, 2000). Instead, *SFB2* was disrupted in a *sec24-1* background. *sec24-1 SFB2* and *sec24-1 sfb2Δ* cells were subjected to pulse-chase experiments at restrictive temperatures and immunoprecipitated with an Hsp150-specific antibody (II, Figure 7). Surprisingly, no differences in the rate of maturation of Hsp150 in these strains were observed. This demonstrates that Sfb2p is not required for Sec24p-independent ER exit of Hsp150. Since SNAREs apparently cannot bind to Sfb3p (Miller *et al.*, 2002), this suggests that the coats contain the temperature-sensitive Sec24p subunit for selection of important SNAREs. This would require that the mutation in Sec24p does not affect binding of SNAREs; this remains to be shown. Whether Sfb3p is a constituent of the COPII coat in *sec24-1 sfb2Δ* cells is not known (Figure 8C).

2. C-terminal domain of Hsp150 harbors determinants guiding Hsp150 to Sec13p- and Sec24p-independent transport routes (I, II)

2.1 Hsp150 variants are not secreted in the absence of functional Sec13p or Sec24p

The determinants required for COPI-independent ER exit of Hsp150 have been demonstrated to reside in the repetitive region of subunit II (Suntio *et al.*, 1999). To study whether the same or different signatures guided Hsp150 to Sec13p- and Sec24p-independent pathways, we followed the fate of several different Hsp150 variants in these mutants using enzymological, biochemical, and morphological techniques.

Three fusion variants were constructed in which the *E. coli* β-lactamase was fused to the C-terminus of differently truncated Hsp150 versions. In Hsp150 subunit I (SUI)-β-lactamase (Figure 6B), the whole subunit II was omitted and β-lactamase was fused directly to subunit I of Hsp150. Hsp150Δ-β-lactamase (Figure 6C) contained subunit I, the entire repetitive region of subunit II, and twenty amino acids from the beginning of the N-terminus of the C-terminal domain, followed by β-lactamase. The third variant, SUI-R3-β-lactamase (Figure 6D), contained subunit I and the first three repeats of subunit II, followed by β-lactamase. All fusion genes were under the control of the *HSP150* promoter.

In wild-type cells, SUI-β-lactamase (Figure 6B) is not secreted but rather targeted to the vacuole for degradation at both 24°C and 37°C (Suntio *et al.*, 1999). To study whether ER exit of SUI-β-lactamase required functional Sec13p and/or Sec24p, we investigated the fate of the protein in *sec13-1* and *sec24-1* mutants at restrictive temperatures by pulse-chase analyses. In case the transport of SUI-β-lactamase occurs independently of functional Sec13p or Sec24p, we should either detect a reduction in signal intensity during the chase due to the degradation of SUI-β-lactamase in the vacuole or, alternatively, a decrease in electrophoretic mobility due to Golgi-specific extensions of O-glycans of subunit I. After a 15-minute preincubation at 37°C, *sec13-1* and *sec24-1* cells were ^{35}S-labeled for three minutes and chased in the presence of CHX for up to 90 minutes at 37°C. SUI-β-lactamase was immunoprecipitated from cell lysates with β-lactamase-specific antiserum and analyzed by SDS-PAGE and autoradiography. No change in signal intensity or electrophoretic mobility was detected (I, Figure 8; II, data not shown). This demonstrates that SUI-β-lactamase is unable to leave the ER in the absence of functional Sec13p or Sec24p.

When Hsp150Δ-β-lactamase (Figure 6C) is expressed in a *SEC*$^{+}$ strain, the β-lactamase portion is correctly folded into an enzymatically active conformation, and the fusion protein is secreted to the culture medium within 20 minutes at 37°C (I, Figure 4B; Simonen *et al.*, 1994; Paunola *et al.*, 1998). Forward transport of this fusion protein is also independent of a functional COPI-mediated pathway (Suntio *et al.*, 1999). If the signatures guiding Hsp150 to COPI- and Sec13p/Sec24p-independent pathways resided in the same repetitive region, Hsp150Δ-β-lactamase should leave the ER also in the

absence of fully functional Sec13- and Sec24-proteins. Hsp150Δ-β-lactamase was expressed in a *sec13-1* strain and its localization was followed by determining intracellular, extracellular, and cell-wall β-lactamase activities during the experiment. At a restrictive temperature (37°C), the β-lactamase activity accumulated solely inside the cells and no activity could be detected in the cell wall or culture medium (I, Figure 4A). CHX was added after one hour to stop protein synthesis, and the cells were shifted back to a permissive temperature (24°C) to return the activity of the mutated Sec13 protein. At the permissive temperature, the intracellular activity of β-lactamase slowly began to decrease, while the activity in the culture medium increased. Since synthesis of new proteins was prevented, the increase in extracellular β-lactamase activity must be due to resumed protein transport after reactivation of temperature-sensitive Sec13p.

In conclusion, activity measurements indicated that Hsp150Δ-β-lactamase was secreted only when Sec13p was functional. Based on these data, two models were formulated. First, Hsp150 could have two different signatures for ER exit, one for COPI-independent secretion in a repetitive region of subunit II, and another for Sec13p-independent secretion in the C-terminal domain. Second, Hsp150Δ-β-lactamase may have been capable of leaving the ER in the investigated *sec13-1* mutant but was trapped in a post-ER organelle before externalization.

2.2 Hsp150 variants remain in the ER in *sec13-1* cells

Pulse-chase analysis of SUI-β-lactamase had shown that it remained in the ER in the absence of functional Sec13p or Sec24p. To identify the organelle in which Hsp150Δ-β-lactamase accumulated in *sec13-1* cells, we used both morphological and biochemical techniques. Indirect immunofluorescence staining of Hsp150Δ-β-lactamase in the *sec13-1* mutant revealed that the fusion protein was localized to structures reminiscent of ER in the absence of functional Sec13p (I, Figure 5).

Biochemical evidence was obtained by a pulse-labeling experiment. As Hsp150Δ-β-lactamase has a Kex2p cleavage site between subunits I and II, processing in this site would suggest arrival in the *trans*-Golgi, where Kex2p is located (Franzusoff *et al.*, 1991). However, the change in electrophoretic mobility is too small to be detected in SDS-PAGE. To be able to distinguish the Kex2p-cleaved form from the uncleaved one, we used SUI-R3-β-lactamase (Figure 6D), in which there are only three repetitive peptides instead of eleven.

sec13-1 cells expressing SUI-R3-β-lactamase were ^{35}S-labeled for three minutes after a 15-minute preincubation at 37°C and chased in the presence of CHX for one hour at 37°C. Part of the cells were shifted to permissive temperature 24°C to release the secretory block but kept in a medium containing CHX for further chase (I, Figure 6). R3-β-lactamase, the Kex2p-cleaved form, was detected from the cell lysates and the medium only after the secretory block was reversed at 24°C. Thus, SUI-R3-β-lactamase was unable to reach the Kex2p-

containing Golgi compartment in the absence of functional Sec13p. SUI-R3-β-lactamase most likely remained in the ER since the form accumulating inside the cells at the restrictive temperature comigrated with the SUI-R3-β-lactamase arrested in the ER in a *sec18-1* mutant (I, Figure 6). Sec18p is a cytosolic protein required for all fusion steps along the secretory pathway. The mutant phenotype detected in *sec18-1* cells at the restrictive temperature is the accumulation of ER and ER-derived vesicles, and as a consequence, ER forms of cargo proteins (Kaiser and Schekman, 1990).

2.3 Hsp150Δ-HRP localizes to ER if Sec24p is defective

To confirm the localization of Hsp150 lacking the C-terminal domain we performed an ultrastructural localization analysis at electron microscopic level. Instead of using indirect immunolabeling, we employed a more sensitive method.

In the presence of hydrogen peroxide (H_2O_2), horseradish peroxidase (HRP) is able to oxidize several substrates. Reaction with 3,3-diaminobenzidine (DAB) results in a brown, highly insoluble DAB polymer at the site of enzyme activity. Further reaction with osmium tetroxide (OsO_4) increases the electron density of the polymer, making it suitable for ultrastructural studies. This technique has been used for immunohistochemical and immunocytochemical localization of mammalian proteins using secondary antibodies conjugated with HRP (Brown and Farquhar, 1989).

A simplified method is to fuse HRP directly to the protein of interest (Connolly *et al.*, 1994), and this has been shown to provide high sensitivity; the detection limit is one molecule in a 60-nm vesicle (Stinchcombe *et al.*, 1995).

Here, we adapted this technique to yeast. To this end, we constructed a new fusion protein. Hsp150Δ-HRP (Figure 6E) is similar to Hsp150Δ-β-lactamase (Figure 6C) except that the C-terminal domain of Hsp150 is replaced with HRP instead of β-lactamase. The fusion gene was expressed from the *HSP150* promoter.

sec24-1 cells grown at an early logarithmic phase at 24°C were first incubated for one hour at a restrictive temperature to impose the mutant phenotype and upregulate the expression of Hsp150Δ-HRP. Cells were then fixed with formaldehyde, treated with DAB/OsO_4, and embedded in an appropriate resin. Finally, thin sections of samples were post-stained with uranyl acetate and lead citrate and examined for HRP reaction product with electron microscopy.

In yeast, the ER is localized both to perinuclear and cortical regions. Perinuclear ER is continuous with the nuclear envelope, whereas cortical ER beneath the plasma membrane is connected to the cell cortex (Preuss *et al.*, 1991; Fehrenbacher *et al.*, 2002). In *sec24-1* cells, stained structures resembling ER were found close to the plasma membrane and inside of the cells (II, Figure 5A).

Hsp150Δ-HRP was also expressed in a *sec23-1* mutant. Nonfunctional Sec23p blocks ER exit of all proteins (including Hsp150) from the ER at restrictive temperatures (Kaiser and Schekman, 1990). Staining was observed inside membranous structures around the nucleus and beneath the plasma

membrane (II, Figure 5C). Again, these ER membranes seemed to be more prominent compared with typical wild-type cells (data not shown). Accumulation of ER membranes often occurs when the formation of ER-derived vesicles is inhibited (Kaiser and Schekman, 1990; Peng *et al.*, 2000). We believe that the structures containing the HRP reaction product in the *sec24-1* mutant were exaggerated ER membranes that resulted from ER retention of Hsp150Δ-HRP.

Hsp150Δ-HRP was also expressed in a *sec7-1* mutant. Sec7p is a GEF for small GTPases Arf1p and Arf2p (Sata *et al.*, 1998), which initiate the assembly of the COPI coat at Golgi membranes (Serafini *et al.*, 1991). Staining was detected in structures resembling stacked Golgi cisternae (II, Figure 5D) similar to those observed earlier in a *sec7-1* mutant stained with OsO_4 (Novick *et al.*, 1981). As a reference, *sec7-1* cells lacking Hsp150Δ-HRP showed reaction product only in the vacuole, which contained endogenous peroxidases (II, Figure 5B).

Taken together, biochemical and morphological evidence indicates that Hsp150 is arrested in the ER in a Sec13p- and Sec24p-dependent fashion when the C-terminal domain of Hsp150 is replaced with a nonrelated fusion partner. Here, we show that the repetitive region alone or in combination with subunit I was insufficient to guide Hsp150 to Sec13p- or Sec24p-independent pathways, dissimilar to COPI-independent transport of Hsp150 (Suntio *et al.*, 1999). Thus, the signal recruiting Hsp150 to Sec13p- and Sec24p-independent transport routes is different from the signal that directs Hsp150 to the COPI-independent anterograde pathway.

2.4 C-terminal domain of Hsp150 is required for ER exit in COPII mutants

As replacing the C-terminus of Hsp150 with β-lactamase or HRP made the protein dependent on both Sec13p and Sec24p, our interest was directed to the role of the C-terminal domain itself. The results proposed that this domain, and not the repetitive region, was important for selection of Hsp150 into COPII vesicles generated in the presence of nonfunctional Sec13p or Sec24p. To investigate this more directly, we transformed two different Hsp150 variants into *sec13-1* and *sec24-1* mutants. The first was Hsp150Δ, a truncated version of Hsp150 lacking the last 89 amino acid residues of the 114-amino acid C-terminal domain (Figure 6F). The second variant was SUI-Cterm, a construct lacking the entire repetitive region but containing an intact C-terminal domain (Figure 6G). According to our hypothesis, the former variant would remain in the ER, while the latter would bypass the ER exit block in the two *sec* mutants at nonpermissive temperatures.

Cells were pulse-labeled and chased like before and then immunoprecipitated with antiserum specific for Hsp150. Both protein variants in *sec13-1* and *sec24-1* mutants at 24°C, as well as in control cells at 24°C and 37°C, were secreted to the culture medium (I, Figures 7 and 8A; II, Figures 3 and 4). Thus, the Hsp150 variants were able to fold into secretion-competent conformations in the assay

conditions. When pulse-labeled Hsp150Δ was immunoprecipitated from a *sec13-1* mutant after a one-hour chase at 37°C, the majority of the truncated protein was detected in the cell lysates comigrating with an ER form of Hsp150Δ immunoprecipitated from a *sec18-1* mutant (I, Figure 7). In *sec24-1* cells, the intracellular retention was quantitative; virtually no Hsp150Δ was secreted to the culture medium at 37°C (II, Figure 4). The reason for "leakiness" of the *sec13-1* mutant is currently unknown. However, the amount of secreted Hsp150Δ also in a *sec13-1* background was always much less than the amount of the ER-arrested form.

Thus, when the C-terminus is missing, Hsp150 cannot leave the ER in the absence of functional Sec13p or Sec24p. Interestingly, as the truncated Hsp150 was secreted at 37°C from a wild-type strain (as well as from COPII mutant strains at 24°C), the results indicate that the C-terminus is important only when the full function of COPII machinery is compromised.

The fate of SUI-Cterm was monitored by similar pulse-labeling experiments. The C-terminal domain was detected in the culture medium both in *sec13-1* (I, Figure 8A; II, Figure 3B) and *sec24-1* (II, Figure 3A) mutants incubated at restrictive temperatures.

Based on these data, we suggest that the determinant(s) of Hsp150 required for Sec13p- or Sec24p-independent transport reside in the 114-amino acid C-terminal domain. This indicates that Hsp150 harbors two distinct signatures: one guiding Hsp150 to the COPI-independent pathway in the repetitive region of subunit II, and (at least) one guiding Hsp150 to Sec13p- and Sec24p-independent routes in the C-terminus.

2.5 Active signal within the C-terminal domain of Hsp150 recruits fusion proteins to Sec13p- and Sec24p- independent ER exit

Here, we addressed the question of whether the C-terminal domain is an active mediator for Sec13p and Sec24p-independent transport, or whether its effect on ER exit is limited to Hsp150 molecules. If the former and more tempting alternative is true, then the C-terminal domain should be capable of guiding other yeast proteins to this alternative ER exit route as well. To answer this, we constructed two new fusion proteins, SUI-invertase-Cterm and SUI-Cterm-invertase (Figure 6H and I).

In SUI-invertase-Cterm, the repetitive region of subunit II was replaced by invertase. We had already confirmed that ER exit of invertase required fully functional Sec13p and Sec24p in the cells in which Hsp150 was secreted (I, Figure 2B; II, Figure 2C). In SUI-Cterm-invertase, the order of fusion partners was inverted such that the C-terminal domain preceded invertase. The authentic Kex2p-cleavage site right after subunit I was maintained in both constructs. This was done to enable monitoring of Kex2p cleavage of the proteins, indicating arrival in the Golgi. This served as direct evidence for exit from the ER. Furthermore, these constructs allowed us to determine whether the orientation of the C-terminus was important. The expression of both recombinant genes was placed under the control of the

HSP150 promoter. Thus, we were able to use 2% glucose throughout the experiment to conveniently repress synthesis of endogenous invertase, and any invertase activity detected would be due to the fusion proteins.

The fusion genes were transformed into *SEC*$^+$, *sec13-1*, *sec24-1*, and *sec18-1* cells. For reference, we first confirmed that at 24°C both fusion proteins were secreted to the cell wall in a catalytically active form in all strains studied (I and II, data not shown). ^{35}S-labeling and chase were conducted as described earlier at 37°C. Samples were immunoprecipitated with invertase-specific antiserum and digested with endoglycosidase H to remove N-glycans from the invertase portion. This simplified analyses of apparent molecular weights of fusion proteins. Endoglycosidase H-treated invertase is detected as a sharp band in SDS-PAGE, whereas nontreated invertase migrates as a smear due to heterogeneously modified N-glycans (Lehle *et al.*, 1979; Esmon *et al.*, 1981).

SUI-invertase-Cterm immunoprecipitated from *sec18-1* cells migrated like a 81-kDa protein, while in wild-type (*SEC*$^+$) cells a protein of 73 kDa was detected (II, Figure 6A). The mobility difference was in agreement with the removal of subunit I (approximately 10 kDa; Suntio *et al.*, 1999) by Kex2p in the late Golgi. Thus, we suggest that the 81-kDa form represents the ER form of SUI-invertase-Cterm, whereas the 73-kDa form is the mature form where SUI is lost.

In *sec13-1* and *sec24-1* mutants at restrictive temperatures, two protein bands were detected, one comigrating with the mature form, and another migrating like a 82-kDa protein (II, Figure 6A). This 82-kDa band most likely represents the uncleaved ER form of SUI-invertase-Cterm. The slightly retarded migration compared with the 81-kDa form found in the *sec18-1* mutant must be due to overglycosylation of the ER-trapped fusion protein (see Section 1.2). We conclude that SUI-invertase-Cterm had exited the ER in the absence of functional Sec13p or Sec24p.

Interestingly, the orientation of the C-terminus appeared to be unimportant. Even if the C-terminal domain was placed in the middle of the fusion protein (SUI-Cterm-invertase), the majority of the recombinant protein was detected in a Kex2p-cleaved form in the *sec13-1* and *sec24-1* mutants at restrictive temperatures (I, Figure 9; II, Figure 6A), and thus, had left the ER.

These results indicate that the C-terminal domain is indeed an active mediator of a novel ER exit pathway, which is independent of functional Sec13p and Sec24p. When fused to the N- or C-terminus of invertase, this domain converted an endogenous yeast protein, normally completely dependent on both Sec13p and Sec24p, into a secretion-competent molecule in conditions where the function of the COPII coat was compromised by mutant Sec13p or Sec24p.

2.5.1 Model for ER exit of Hsp150 in the absence of functional Sec13p or Sec24p

Our results demonstrate that although the anterograde transport from the ER to the Golgi is seemingly halted if COPII component Sec13p or Sec24p is defective, the budding of vesicles from the ER membrane is not completely

inhibited. Two pieces of evidence support the secreted Hsp150 being transported *via* the Golgi. First, the portion trapped in the ER always migrates faster than the secreted form in SDS-PAGE. It is thus unlikely that secreted Hsp150 gains all Golgi-specific elongations in the ER within the incubation period. Second, the Kex2p-specific cleavage of SUI-invertase-Cterm and SUI-Cterm-invertase confirms that these recombinant proteins must have been transported beyond the ER: SUI-R3-β-lactamase, which accumulates in the ER in COPII mutants, is not cleaved by Kex2p until the secretory block is reversed (I, Figure 6). Thus, Kex2p cannot accumulate in the ER in the *sec13-1* and *sec24-1* mutants.

Putative receptor recognizes the C-terminal domain of Hsp150

While the ER exit of Hsp150 in wild-type conditions does not require the C-terminal domain, this domain is necessary for ER exit in conditions where the formation of COPII vesicle is compromised. Furthermore, the C-terminal domain is sufficient for conferring Sec13p and Sec24p independence to invertase, which would be retained in the ER if these COPII components were not functional. That strongly suggests that export of Hsp150 from the ER in these circumstances is an active process, *i.e.* requires selection.

VSV G, mammalian potassium channels Kir1.1 and Kir1.2, as well as yeast Sys1p and Gap1p, are all proteins that harbor a cytosolic diacidic ER export signal required for efficient packaging of these proteins into COPII vesicles (Nishimura and Balch, 1997; Ma *et al.*, 2001; Votsmeier and Gallwitz, 2001; Malkus *et al.*, 2002). Apparently, all of these proteins are selected to the vesicles *via* direct interactions between the diacidic signal and components of the COPII coat (Aridor *et al.*, 1998; Miller *et al.*, 2003). On the other hand, selection of several type I integral membrane proteins (p24 family, ERGIC-53, Emp46p, Emp47p) into COPII vesicles depends on a dihydrophobic motif at the C-terminus (Dominguez *et al.*, 1998; Nufer *et al.*, 2002; Sato and Nakano, 2002). The motif in some p24 family members has been shown to interact directly with the coat subunits (Dominguez *et al.*, 1998), and it is likely that all proteins with a dihydrophobic signal are selected in a similar fashion. In addition, the binding sites for three SNARE proteins have been characterized in Sec24p (Miller *et al.*, 2003; Mossessova *et al.*, 2003).

However, not all transmembrane proteins can bind directly to COPII components. The family of p24 proteins (Emp24p, Erv25p, and Erp1-Erp6p in yeast; Marzioch *et al.*, 1999) recycles between the ER and the Golgi and has been proposed to function as cargo receptors for secretory proteins. One member of this family, Emp24p, has been shown to recruit Gas1p, a GPI-anchored plasma membrane protein, into COPII vesicles (Muñiz *et al.*, 2000). However, deletion of all eight p24 family members in yeast is not lethal (Springer *et al.*, 2000), demonstrating that they are not essential in protein transport. Another regular constituent of the ER-derived vesicles, Erv14p, is required for selecting Axl2p, a plasma membrane protein, to the COPII coat. Erv14p has been shown to bind to both Axl2p and the prebudding complex of COPII (Powers and Barlowe, 2002). Yet another example of a protein

that enhances cargo selection of other proteins is Shr3p, a multispanning membrane protein located at the ER membrane. It is specifically required for packaging of amino acid permeases into transport vesicles, although the protein itself is excluded from the vesicles (Gilstring *et al.*, 1999).

These are all examples of membrane proteins that by interacting directly or indirectly with COPII components are selected to the forming vesicles. However, Hsp150 is a soluble protein in the lumen of the ER. This suggests that Hsp150 is recognized by another protein, a cargo receptor, which in turn interacts with the components of the COPII coat either directly or indirectly.

Serum albumin has been reported to concentrate at ER exit sites (Mizuno and Singer, 1993), although apparently not all soluble proteins behave similarly (Martínez-Menárguez *et al.*, 1999). However, it is quite evident that at least two cargo receptors for soluble proteins exist. ERGIC-53, a mammalian lectin, binds transiently to certain glycoproteins and transports them to post-ER organelles (Appenzeller *et al.*, 1999). The yeast homologs of ERGIC-53, Emp46p, and Emp47p also appear to function in glycoprotein secretion (Sato and Nakano, 2002). The second cargo receptor identified is Erv29p, an integral membrane protein recycling between the ER and Golgi, and it is required for

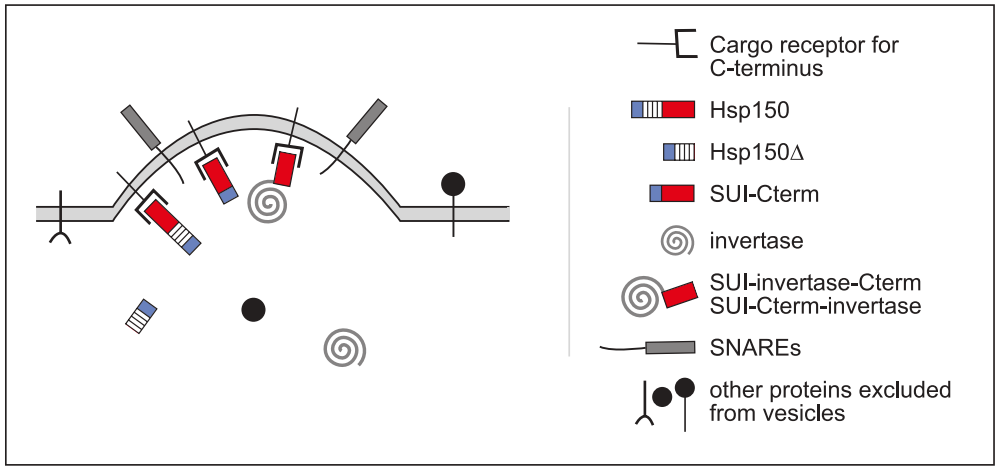


Figure 9. Model for a putative cargo receptor specific for the C-terminal domain of Hsp150. When the vesicle formation is driven in the presence of nonfunctional Sec13p or Sec24p, ER exit of Hsp150 becomes dependent on the C-terminal domain. This domain is also capable of recruiting normally Sec13p- and Sec24p-dependent invertase into these specific vesicles. COPII components are omitted for clarity.

efficient packaging of glycosylated pro-α-factor into ER-derived vesicles (Belden and Barlowe, 2001b).

Based on the active role of the C-terminal domain of Hsp150 in selection of proteins containing this domain into COPII vesicles, most likely a protein is present in the ER that has an affinity towards the C-terminus of Hsp150. As Hsp150 lacking the C-terminal domain is secreted in normal conditions, this putative receptor is not essential for Hsp150 selection if the function of COPII is not compromised. However, in *sec13-1* and *sec24-1* mutants at restrictive temperatures the ER exit of Hsp150 or its variants depends on the C-terminal domain, and therefore, on the interaction between the receptor and the C-terminal domain. The nature of the putative cargo receptor has not been studied to date. It could be an as yet unidentified integral membrane protein, or alternatively, one of the SNAREs. Furthermore, the role of possible lipid interactions cannot be excluded. A model of recognition of the C-terminal domain of Hsp150 by a putative cargo receptor at the ER exit site is presented in Figure 9.

3. Yeast has three members in the family of BAP31-like proteins (III)

3.1 A new protein family, the BAP31-like proteins

Mammalian BAP31 is a type I integral membrane protein with three transmembrane domains (TMDs) located at the ER membrane (Annaert *et al.*, 1997; Ng *et al.*, 1997). Several studies suggest that it has an active role in apoptosis: when one or both caspase cleavage sites in the C-terminal tail of BAP31 are processed, the protein is capable of inducing apoptosis in living cells (Ng *et al.*, 1997; Granville *et al.*, 1998; Ng and Shore, 1998; Määttä *et al.*, 2000). However, another role for BAP31 has also been suggested – a role that, at least based on present knowledge, seems to be unrelated to apoptosis. Evidence from this line of studies suggests that BAP31 enhances the ER exit of several proteins, perhaps in a form of a cargo receptor (Annaert *et al.*, 1997; Spiliotis *et al.*, 2000; Lambert *et al.*, 2001). We decided to investigate whether the yeast proteins homologous to BAP31 might participate in the ER exit of other proteins by acting as cargo receptors.

BLAST searches revealed several genes coding for putative BAP31 homologs in databases of various organisms, including humans, mice, fruit flies, nematodes, baker's yeast, fission yeast, zebrafish, and thale cress (III, Figure 1 and data not shown). Sequence analyses showed that the proteins, true or hypothetical, had several characteristics in common. First, the length of the proteins did not vary markedly. The shortest protein encoded was 160 amino acids (*S. cerevisiae* Yet2p), and the longest was 247 amino acids [a hypothetical open reading frame (ORF) in zebrafish]. Second, TMD prediction programs suggested three TMDs for each protein, within approximately the first 120 amino acids of the protein. Variations in length are therefore concentrated in the C-terminal, cytosolic portion. Third, we noticed that in each protein one or two of these TMDs contained charged amino

acids. Fourth, a canonical short peptide was similarly located in all protein sequences: just preceding and in the second predicted TMD. Fifth, 10 of 12 sequences had a C-terminal ER retrieval motif (KKXX). Type I membrane proteins containing this motif are usually localized to the ER. The motif was first characterized as an ER-retention motif in mammalian cells (Nilsson *et al.*, 1989; Jackson *et al.*, 1990) and in yeast (Gaynor *et al.*, 1994; Townsley and Pelham, 1994). Later, chimera proteins containing the KKXX motif were shown to receive Golgi-specific modifications, suggesting that this motif conveys retrieval as well as retention information (Jackson *et al.*, 1993). Finally, the C-terminal portions of all putative proteins discovered had potential to form coiled-coil structures (III, Figure 1 and data not shown).

We suggested that these 12 true or putative proteins found from eight different organisms form a new family of BAP31-like proteins. Three members of this family, designated Yet1p-Yet3p (see below), were found in yeast *S. cerevisiae* (III, Figure 1). Although the overall sequence identity between BAP31 and the yeast proteins was not very high, based on similar structural features, the proteins were placed in the same family. Yet1p has 30%, Yet2p 22%, and Yet3p 26% identity in amino acid sequence compared with BAP31, whereas the average sequence identity between all family members is 43%, implicating a higher degree of conservation between higher eukaryotes.

Since many basic biological processes are conserved between yeast and higher eukaryotes, it is reasonable to consider whether the properties and functions of a particular protein in one organism might be preserved in its orthologs. Given the various functions of mammalian BAP31, we made an attempt to shed light on the possible conserved functions of this protein family.

3.2 *YET* genes encode proteins expressed in yeast cells

Search for BAP31 homologs in *Saccharomyces* Genome Database (SGD) revealed three ORFs, YKL065C, YMR040W, and YDL072C, which coded for hypothetical proteins of 206, 160, and 203 amino acids, respectively. Northern analysis of total RNA proved that YKL065C gene was transcriptionally active (III, data not shown). Polyclonal antibodies were raised against the C-terminal tail of the protein encoded by YKL065C and the expression of the protein was verified by Western analysis of yeast cell lysates (III, Figure 2). The gene was designated *YET1* (for encoding a yeast ER transmembrane protein).

Two pieces of evidence suggested that the YMR040W gene was silent: it could not be cloned from a cDNA library, and the corresponding mRNA was not detected in Northern analysis (III, data not shown). However, inspection of the growth phenotypes of the strains from which YMR040W was deleted revealed that the gene was not completely silent (see Section 3.4.1). This ORF was designated *YET2*.

The third ORF, YDL072C, did not appear in the first homology screen but was found later in BLAST searches with a partial BAP31 amino acid sequence. The localization of the protein encoded by YDL072C was determined in a genome-wide localization analysis of

yeast proteins (Huh *et al.*, 2003). In that study, the researchers made a collection of yeast strains, with each containing an ORF tagged with a green fluorescent protein (GFP) in the C-terminus of the ORF. All ORF-GFP fusion genes were under the control of the promoter of the ORF in question. Thus, the positive signal of YDL072C-GFP also indicated that the endogenous YDL072C was actively transcribed and translated. Because this protein appeared to be the third yeast member of the BAP31-like proteins (III, Figure 1), the YDL072C gene was designated *YET3*.

3.3 Subcellular localization of Yet proteins

Biochemical studies of BAP31 confirmed the characteristics predicted by sequence analysis programs: BAP31 is an ER-localized integral membrane protein with type I topology and no cleavable signal sequence (Annaert *et al.*, 1997; Ng *et al.*, 1997). Type I proteins located at the ER membrane have their N-terminus in the lumen; if the protein has three TMDs, its C-terminal tail would point to the cytosol. For BAP31, this orientation confers correct localization of the KKXX motif on the cytosolic side.

3.3.1 Biochemical properties and evidence for ER localization of Yet1p

Sequence analysis programs suggested that Yet1p, like all other BAP31-like proteins, has three TMDs but no cleavable signal sequence. To confirm that the signal sequence of Yet1p was not cleaved, the protein was translated *in vitro*. Proteins synthesized in either the presence or the absence of microsomes comigrated in SDS-PAGE, suggesting that the first hydrophobic segment was not cleaved but had probably served as the first TMD (III, data not shown).

Membrane association can be investigated by analyzing the pattern of fractionation of a protein into high-speed pellet and supernatant fractions after centrifugation of cell lysate at 100,000 *g*. Membrane-associated proteins fractionate to the pellet fraction, whereas soluble proteins remain in the supernatant. Peripheral membrane proteins, which are often associated with the membranes through electrostatic interactions with other membrane proteins or phospholipids, can be released to the supernatant fraction by disrupting these interactions with various agents. Integral membrane proteins, by contrast, can be liberated from the membranes only by detergent treatment.

Isolated yeast membranes were incubated in the presence of the following reagents known to release peripheral membrane proteins: high salt, high pH, and urea. None of these was able to solubilize Yet1p into the supernatant fraction (III, Figure 3). Only detergent (1% Triton X-100) partially released Yet1p, indicating that the protein was associated with the membranes through hydrophobic interactions.

Next, the localization of Yet1p was studied by two different techniques, indirect immunofluorescence and subcellular fractionation (III, Figure 4). In immunofluorescent microscopy, cells overproducing Yet1p were used because the signal obtained by the Yet1p-antibody of the endogenous protein was faint. For reference, another set of cells was stained with an antibody specific to Kar2p, a

known ER resident chaperone (Normington *et al.*, 1989; Rose *et al.*, 1989). Both antibodies stained membraneous structures reminiscent of perinuclear ER as well as membranes beneath the plasma membrane corresponding to cortical ER (III, Figure 4A).

For subcellular fractionation, yeast membranes were prepared from wild-type cells and fractionated by velocity sedimentation on a step-wise 22-60% sucrose density gradient. With a similar technique, an ER-localized protein (Wbp1p) has previously been shown to sediment to the bottom fractions, whereas Golgi markers (Pmr1p, Och1p, GDPase, Kex2p) were detected in the middle fractions and a vacuolar protein (vacuolar ATPase) in the top (light) fractions (Schröder *et al.*, 1995). After sedimentation, Yet1p together with Seb1p, an integral ER membrane protein (Toikkanen *et al.*, 1996), and Sso2p, a plasma membrane protein (Aalto *et al.*, 1993), were immunodetected in each fraction by Western analysis (III, Figure 4B). Yet1p and Seb1p co-fractionated to bottom fractions, whereas Sso2p was completely separated from them, occupying the lightest fractions.

Our results obtained by two independent techniques, the existence of an ER-retrieval motif in the C-terminus of Yet1p, and a recent global study of yeast protein localization (Huh *et al.*, 2003) confirm that Yet1p is localized to the ER membrane.

3.2.2 Localization of Yet2p and Yet3p

Biochemical characterization of Yet2p was not conducted due to the assumption that the gene is silent (see Section 3.2). In addition, evidence for location of Yet2p was not obtained from the genome-wide localization study of yeast proteins, although all annotated ORFs were included (Huh *et al.*, 2003). Of the 6234 ORFs, roughly 30% (2078) were excluded due to problems in the cloning phase or low GFP signal levels. *YET2* must have been one of the ORFs that could not be analyzed. The reason it could not be analyzed remains unknown to us. However, the studies conducted with *yet2* deletion strains argue that absence of the protein product affected yeast growth when *YET3* was deleted, indicating that Yet2p must perform some function in yeast cells (see Section 3.4.1).

YET2 was, however, included in a study where the topology of 37 yeast integral membrane proteins was experimentally determined (Kim *et al.*, 2003). A fusion partner consisting of partial *SUC2* and *HIS4* genes was fused to the C-terminus of the selected ORFs and the resulting fusion genes were expressed from the constitutive *TPI* promoter.

Evidence for cytosolic localization of the C-terminus of Yet2p was obtained in two ways. The strain (*his4-401*) carrying the *YET2-SUC2-HIS4* fusion gene was analyzed for growth after histidinol was added (Kim *et al.*, 2003). *HIS4* encodes the enzyme that converts histidinol to histidine, an essential amino acid for yeast. The strain containing the Yet2p chimera was able to grow when provided with histidinol, indicating that the His4 portion was located in the cytosol. In addition, the Suc2 portion in the C-terminal tail of the fusion protein was not glycosylated. This supports the view that the tail was facing the cytosol. With three predicted TMDs, the authors concluded that Yet2p is a type I integral membrane

protein (Kim *et al.*, 2003). The article does not, unfortunately, provide evidence of the activity of *YET2* promoter because of the use of the *TPI* promoter, nor does it depict the intracellular location of Yet2p.

The only indication of the location of Yet2p can be obtained from the amino acid sequence: the C-terminal ER retrieval motif suggests localization at the ER membrane. However, two type I integral membrane proteins containing the retrieval motif, namely Emp47p and related Emp46p, have been shown to localize to the Golgi (Schröder *et al.*, 1995; Sato and Nakano, 2002). Hence, the ER retrieval motif of Yet2p can be considered a suggestion, not a verification of localization in the ER.

As described in Section 3.2, the localization of *YET3* was analyzed in a global yeast protein analysis (Huh *et al.*, 2003). Based on fluorescence microscopy of the Yet3-GFP fusion protein, Yet3p was categorized as an ER protein. Interestingly, although the GFP tag in the C-terminus abolishes activity of the ER retrieval motif, the protein was still detected in the ER in that study. Similar observations have been made with the mammalian BAP31; deletion of the KKXX motif does not alter the ER localization of the protein (Annaert *et al.*, 1997). This indicates that these proteins might possess additional signals to guarantee ER retention.

3.4 Disruption of *YET1* or *YET3* shows opposite effects on yeast growth

A simple method to characterize the role of a specific protein is deletion of the respective gene from the genome and then investigation of whether any mutant phenotype exists. With yeast, individual genes can conveniently be deleted or additional genes introduced into desired locuses by homologous recombination. This makes yeast a popular organism for functional studies.

Wild-type yeast cells do not have to be provided with amino acids because they are able to synthesize all essential amino acids themselves. However, if a gene involved in a biosynthesis of some amino acid is nonfunctional (*i.e.* due to a mutation), the strain is referred to as auxotrophic for that amino acid. Consequently, the amino acid in question has to be supplemented in the growth medium for the yeast to grow. This property of yeast is routinely taken advantage of when, for example, disrupting desired genes: if a yeast strain has a mutation in the *URA3* gene, for instance, which is one of the enzymes required for biosynthesis of uracil, it cannot grow on media lacking uracil. Then, a cassette containing an intact *URA3* gene and all the elements required for its proper expression (promoter and terminator regions) flanked by sequences homologous to upstream and downstream regions of the gene to be disrupted is introduced into the yeast genome harboring the *ura3* mutation. By homologous recombination, the DNA fragment is directed to the correct locus and the gene of interest is replaced. Thereby, the ability to synthesize uracil is restored. In addition to uracil, the biosynthesis of several other amino acids can be disrupted in the same way. This gives access to a wide selection of auxotrophic markers in a single yeast strain.

In addition to auxotrophic markers, a variety of other markers selectable by dominant resistance have also been developed for yeast. The *kan*^{r} gene of *E. coli* transposon *Tn*903, which encodes aminoglycoside phosphotransferease, confers *E. coli* resistance to the antibiotic kanamycin and yeast *S. cerevisiae* resistance to the aminoglycoside G418 (Jimenez and Davies, 1980). Similarly to auxotrophic disruption cassettes, a cassette designed to express the *kan*^{r} gene in yeast can be used for directed disruption of yeast genes.

Eighty percent of the coding sequence of *YET1* was disrupted with the *URA3* cassette, whereas *YET2* and *YET3* were entirely deleted from the genome with a KanMX4 module (giving resistance to G418) and a *LEU2* cassette (restoring biosynthesis of leucine), respectively. When the strains with single *yet* disruptions were grown on plates on rich or defined media, no differences were observed with various carbon sources or at temperatures ranging from 34°C to 38°C (III, data not shown). Strains harboring all combinations of disruptions were then constructed; three double disruptant strains (*yet1Δ yet2Δ*, *yet1Δ yet3Δ*, *yet2Δ yet3Δ*) and the triple disruptant (*yet1Δ yet2Δ yet3Δ*). Again, no mutant phenotype was detected when grown on plates (III, data not shown).

To obtain a more detailed analysis of growth, the growth rates of each strain were determined in liquid cultivations at 30°C. A bit unexpectedly, clear differences were obtained with this method (III, Figure 5A). Disruption of *YET1* gave cells a growth advantage, whereas absence of Yet3p retarded their growth. When specific growth rates were determined for the exponentially growing strains (calculated from cell densities at four and eight hours from the beginning of the experiment), *yet1Δ* cells were observed to grow slightly (1.5 times) faster than parental cells. The effect of *yet3* disruption was more severe; growth was five times slower than in the parental strain (III, Figure 5B). Single deletion of *YET2* had no effect (III, Figure 5A and B).

3.4.1 Evidence supporting expression from YET2 gene

Inspection of the growth rates of the double disruptant strains revealed that the *YET2* gene could not be silent. Deletion of *YET2* had no effect on *yet1Δ* background, *i.e*. the strain behaved similarly to the one from which *YET1* alone had been disrupted. However, the *yet2Δ yet3Δ* strain grew like the parental strain (III, Figure 5B). This shows that the slow growth caused by deletion of *YET3* was suppressed by simultaneous deletion of *YET2*. In other words, absence of Yet2p was beneficial to cells lacking Yet3p.

By some unknown mechanism, the Yet proteins affect cell growth; Yet1p and Yet2p appear to have a negative effect since the absence of these proteins enhances growth. In contrast, because cells lacking Yet3p grow more slowly, this protein has a positive effect on growth. The effect of *YET2* deletion was detectable only in a *yet3Δ* background. This could indicate that either *YET2* is not constitutively expressed or its absence is compensated by other Yet proteins.

Although the functions of Yet proteins may be partially redundant, the growth defect in *yet3Δ* strain shows that these proteins might also have specialized functions in the cell.

3.5 Secretory pathway functions normally in *yet3* disruptant

Our original attempt was to shed light on the functions of the family of BAP31-like proteins by characterizing the yeast members of this family. We hypothesized that since the structural domains and elements in the amino acid sequences shared by these family members were conserved, their functions would also be at least partially conserved. The mammalian BAP31 is the best-characterized member of the family. It has been implicated in two seemingly unrelated processes in the mammalian cell: apoptosis (Ng *et al.*, 1997; Granville *et al.*, 1998; Ng and Shore, 1998; Määttä *et al.*, 2000) and enhancement of ER exit of a subset of proteins (Annaert *et al.*, 1997; Spiliotis *et al.*, 2000; Lambert *et al.*, 2001). Until quite recently, it has been widely accepted that yeasts do not undergo apoptosis. The benefit obtained by suicide is difficult to explain since yeast is a unicellular eukaryote. Moreover, the yeast genome does not contain ORFs encoding putative apoptosis-related proteins. However, a protein possessing caspase activity has recently been identified in yeast (Madeo *et al.*, 2002), and it now appears that yeast cells also undergo an apoptosis-like cell death (reviewed in Burhans *et al.*, 2003).

A potential role of Yet proteins in ER exit of other proteins appeared, by contrast, to be more probable. As also the slow growth phenotype of *yet3Δ* cells could be envisioned to be a result of a defective secretory process, we decided to compare the maturation kinetics of yeast proteins in *yet1Δ*, *yet3Δ*, and parental strains. Hsp150, CPY, and Gas1p were analyzed by pulse-chase experiments. Secretion of invertase was assayed by activity measurements. Unfortunately, no reproducible differences were observed in the rates of maturation of any of the proteins studied (III, data not shown). Thus, based on these experiments, no evidence has emerged to support the proposed role of Yet proteins in enhancing ER exit of other proteins. Whether Yet proteins assist in transport of proteins that were not included, remains to be studied. However, at this stage it is as possible that Yet proteins function in a process that is completely unlinked to secretion.

CONCLUDING REMARKS

The present study shows that the C-terminal domain of Hsp150 contains a determinant that is able to direct Hsp150 and chimera proteins containing the domain into Sec13p- and Sec24p-independent ER exit pathways. The importance of this determinant becomes evident only under conditions in which formation of transport vesicles is compromised due to nonfunctional Sec13p or Sec24p.

Since Hsp150 is a soluble protein, it cannot directly interact with the COPII components believed to select the cargo for a growing vesicle. However, the C-terminal-dependent recruitment of the protein into COPII vesicles indicates that Hsp150 is actively selected at the ER exit site. This suggests the existence of a protein, possibly a cargo receptor, which has affinity for the C-terminal domain of Hsp150. Interaction between the C-terminal domain and the cargo receptor is essential for efficient packaging of Hsp150 when the vesicles are generated in the absence of functional Sec13p or Sec24p. The nature of the putative cargo receptor was not investigated in this study.

A new family of proteins homologous to mammalian BAP31 is also presented. BAP31 has been suggested, among other things, to function as a cargo receptor at the ER membrane for a subset of proteins. Characterization of the yeast members of this family revealed that two of them, Yet1p and Yet3p, affected the growth rate of yeast when disrupted. Unfortunately, these phenotypes could not be confirmed to result from impaired ER exit of a selection of proteins due to lack of Yet proteins.

The data presented in this study support the view that soluble cargo proteins are actively sorted in the first vesicular step of the secretory pathway. In addition, the results corroborate earlier studies suggesting that multiple, partially redundant mechanisms exist for ensuring cargo selection.

ACKNOWLEDGEMENTS

This work was carried out at the Institute of Biotechnolgy and in the Department of Biosciences, Division of Biochemistry, University of Helsinki. Financial support was provided by the University of Helsinki, the Viikki Graduate School in Biosciences, the Sigrid Jusélius foundation, and the Magnus Ehrnrooth foundation.

I would like to express my profound gratitude to my supervisor, Professor Marja Makarow. Her enthusiasm for science and her enormous effectiveness are exemplary to anyone. Her support and trust in me have been invaluable during this project. I would especially like to thank her for her support during the writing phase of this thesis. She has not only managed to accommodate herself to my stringent schedule in reading my text but she has always been ready to encourage me at times of desperation. In this context I also wish to thank Professor Leevi Kääriäinen for helping me to set the timetable in the final stages of my dissertation. Without Marja, I would never have started to write this book, and without Leevi, I would probably still be writing it.

I am thankful to Professor Mart Saarma, the director of the Institute of Biotechnology, for providing excellent working facilities and influencing in the dynamic and nonhierarchical atmosphere of the institute by his own example. Professor Carl G. Gahmberg, the head of the Division of Biochemistry, is thanked for his positive attitude towards my studies. I wish to thank Professor Timo Korhonen, the head of the Graduate School in Microbiology, for an opportunity to join the Graduate School system. I am deeply grateful to Docents Varpu Marjomäki and Johanna Myllyharju for highly constructive comments on this manuscript.

I thank all the former and present members of the yeast group for creating a warm and an absolutely cheerful spirit in the lab – it could be heard five floors down! Anna Liisa Nyfors is warmly thanked for her excellent technical skills and assistance. During these eight years many people have come and gone; it has been comforting that Anna-Liisa has always been there. To Taina, Hanna, Leena, Monica, Ricardo, Sergei, Laura, Mari, Eeva, Anton, Vijay, Maria, Juha, Qiao, Ansku, Nina S., Heidi, and Eija: thank you for invaluable help in the science related issues as well as for your friendship. There are many places I would probably have missed (like DTM) if it weren't for you. I would like to mention Hanna, who has been in the same phase in writing: it is nice to be able to share the ups and downs in this final project. Eeva: thank you for both spiritual (in form of advice whenever needed and almost on any subject required) and material (in form of baby clothes) support. I would especially like to thank Eija for her friendship outside the lab. I warmly thank the computer support group, Iikka and Petri, for being always ready to help me with all imaginable computer problems I have encountered during these years. Tinde is acknowledged for a nice job in preparing the pictures in this thesis as well as doing the layout.

I am grateful to Docent Esa Kuismanen for taking me to his lab as a laudatur student and guiding me towards PhD studies. It has been a pleasure to see his enthusiasm and dedication to science. I thank Pekka, Memmu, Saara, and Anne

for their friendship and guidance in the first steps of my PhD studies. I also wish to thank my yet unmentioned co-authors, Eija Jokitalo, Jaana Toikkanen, and Sirkka Keränen, whose contribution to two of the studies in this thesis has been invaluable. Jaana I thank also for her friendship. I warmly thank the members of YKEM for welcoming me to their community for a one-year-period. I look forward to working with you again.

I wish to thank all my friends outside the lab. Especially Anne and Tuitu: I am very fortunate in life to have friends like you. I thank Nina, who despite the distance has always remained my friend, for the shared time and discussions.

I owe my special thanks to my mother Raili for her endless love and trust in me. Finally, I wish to express my dearest and deepest gratitude to my fiancé Masa and our son Elia. In the end, it is their love and support that have enabled one of my dreams to come true.

Espoo, May 2004

REFERENCES

Aalto, M. K., Ronne, H., and Keränen, S. 1993. Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. *EMBO J.* **12**, 4095-4104.

Adisa, A., Rug, M., Foley, M., and Tilley, L. 2002. Characterisation of a δ-COP homologue in the malaria parasite, *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **123**, 11-21.

Albano, F. R., Berman, A., La Greca, N., Hibbs, A. R., Wickham, M., Foley, M., and Tilley, L. 1999. A homologue of Sar1p localises to a novel trafficking pathway in malaria-infected erythrocytes. *Eur. J. Cell Biol.* **78**, 453-462.

Annaert, W. G., Becker, B., Kistner, U., Reth, M., and Jahn, R. 1997. Export of cellubrevin from the endoplasmic reticulum is controlled by BAP31. *J. Cell Biol.* **139**, 1397-1410.

Antonny, B., Beraud-Dufour, S., Chardin, P., and Chabre, M. 1997. N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange. *Biochemistry* **36**, 4675-4684.

Antonny, B., Madden, D., Hamamoto, S., Orci, L., and Schekman, R. 2001. Dynamics of the COPII coat with GTP and stable analogues. *Nat. Cell Biol.* **3**, 531-537.

Appenzeller, C., Andersson, H., Kappeler, F., and Hauri, H. P. 1999. The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat. Cell Biol.* **1**, 330-334.

Aridor, M. and Balch, W. E. 2000. Kinase signaling initiates coat complex II (COPII) recruitment and export from the mammalian endoplasmic reticulum. *J. Biol. Chem.* **275**, 35673-35676.

Aridor, M., Fish, K. N., Bannykh, S., Weissman, J., Roberts, T. H., Lippincott-Schwartz, J., and Balch, W. E. 2001. The Sar1 GTPase coordinates biosynthetic cargo selection with endoplasmic reticulum export site assembly. *J. Cell Biol.* **152**, 213-229.

Aridor, M., Weissman, J., Bannykh, S., Nuoffer, C., and Balch, W. E. 1998. Cargo selection by the COPII budding machinery during export from the ER. *J. Cell Biol.* **141**, 61-70.

Baker, D., Hicke, L., Rexach, M., Schleyer, M., and Schekman, R. 1988. Reconstitution of *SEC* gene product-dependent intercompartmental protein transport. *Cell* **54**, 335-344.

Balch, W. E., Dunphy, W. G., Braell, W. A., and Rothman, J. E. 1984. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell* **39**, 405-416.

Barlowe, C., d'Enfert, C., and Schekman, R. 1993. Purification and characterization of SAR1p, a small GTP-binding protein required for transport vesicle formation from the endoplasmic reticulum. *J. Biol. Chem.* **268**, 873-879.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., and Schekman, R. 1994. COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell* **77**, 895-907.

Barlowe, C. and Schekman, R. 1993. *SEC12* encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature* **365**, 347-349.

Belden, W. J. and Barlowe, C. 2001a. Distinct roles for the cytoplasmic tail sequences of Emp24p and Erv25p in transport between the endoplasmic reticulum and Golgi complex. *J. Biol. Chem.* **276**, 43040-43048.

Belden, W. J. and Barlowe, C. 2001b. Role of Erv29p in collecting soluble secretory pro-

teins into ER-derived transport vesicles. *Science* **294**, 1528-1531.

Bi, X., Corpina, R. A., and Goldberg, J. 2002. Structure of the Sec23/24-Sar1 prebudding complex of the COPII vesicle coat. *Nature* **419**, 271-277.

Boehm, J., Letourneur, F., Ballensiefen, W., Ossipov, D., Démollière, C., and Schmitt, H. D. 1997. Sec12p requires Rer1p for sorting to coatomer (COPI)-coated vesicles and retrieval to the ER. *J. Cell. Sci.* **110**, 991-1003.

Boehm, J., Ulrich, H. D., Ossig, R., and Schmitt, H. D. 1994. Kex2-dependent invertase secretion as a tool to study the targeting of transmembrane proteins which are involved in ER → Golgi transport in yeast. *EMBO J.* **13**, 3696-3710.

Brown, W. J. and Farquhar, M. G. 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. *Methods Cell Biol.* **31**, 553-569.

Burhans, W. C., Weinberger, M., Marchetti, M. A., Ramachandran, L., D'Urso, G., and Huberman, J. A. 2003. Apoptosis-like yeast cell death in response to DNA damage and replication defects. *Mutat. Res.* **532**, 227-243.

Burri, L. and Lithgow, T. 2004. A complete set of SNAREs in yeast. *Traffic* **5**, 45-52.

Carlson, M. and Botstein, D. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted with intracellular forms of yeast invertase. *Cell* **28**, 145-154.

Chen, E. J. and Kaiser, C. A. 2003. *LST8* negatively regulates amino acid biosynthesis as a component of the TOR pathway. *J. Cell Biol.* **161**, 333-347.

Connolly, C. N., Futter, C. E., Gibson, A., Hopkins, C. R., and Cutler, D. F. 1994. Transport into and out of the Golgi complex studied by transfecting cells with cDNAs encoding horseradish peroxidase. *J. Cell Biol.* **127**, 641-652.

Cosson, P. and Letourneur, F. 1994. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science* **263**, 1629-1631.

Dascher, C., Ossig, R., Gallwitz, D., and Schmitt, H. D. 1991. Identification and structure of four yeast genes (*SLY*) that are able to suppress the functional loss of *YPT1*, a member of the *RAS* superfamily. *Mol. Cell. Biol.* **11**, 872-885.

d'Enfert, C., Barlowe, C., Nishikawa, S., Nakano, A., and Schekman, R. 1991a. Structural and functional dissection of a membrane glycoprotein required for vesicle budding from the endoplasmic reticulum. *Mol. Cell. Biol.* **11**, 5727-5734.

d'Enfert, C., Gensse, M., and Gaillardin, C. 1992. Fission yeast and a plant have functional homologues of the Sar1 and Sec12 proteins involved in ER to Golgi traffic in budding yeast. *EMBO J.* **11**, 4205-4211.

d'Enfert, C., Wuestehube, L. J., Lila, T., and Schekman, R. 1991b. Sec12p-dependent membrane binding of the small GTP-binding protein Sar1p promotes formation of transport vesicles from the ER. *J. Cell Biol.* **114**, 663-670.

Dominguez, M., Dejgaard, K., Fullekrug, J., Dahan, S., Fazel, A., Paccaud, J. P., Thomas, D. Y., Bergeron, J. J., and Nilsson, T. 1998. gp25L/emp24/p24 protein family members of the *cis*-Golgi network bind both COP I and II coatomer. *J. Cell Biol.* **140**, 751-765.

Donaldson, J. G., Cassel, D., Kahn, R. A., and Klausner, R. D. 1992. ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatomer protein β-COP to Golgi membranes. *Proc. Natl. Acad. Sci. USA* **89**, 6408-6412.

Duden, R., Griffiths, G., Frank, R., Argos, P., and Kreis, T. E. 1991. β-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell* **64**, 649-665.

Elrod-Erickson, M. J. and Kaiser, C. A. 1996. Genes that control the fidelity of endoplasmic reticulum to Golgi transport identified as suppressors of vesicle budding mutations. *Mol. Biol. Cell* **7**, 1043-1058.

Esmon, B., Novick, P., and Schekman, R. 1981. Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. *Cell* **25**, 451-460.

Espenshade, P., Gimeno, R. E., Holzmacher, E., Teung, P., and Kaiser, C. A. 1995. Yeast *SEC16* gene encodes a multidomain vesicle coat protein that interacts with Sec23p. *J. Cell Biol.* **131**, 311-324.

Fehrenbacher, K. L., Davis, D., Wu, M., Boldogh, I., and Pon, L. A. 2002. Endoplasmic reticulum dynamics, inheritance, and cytoskeletal interactions in budding yeast. *Mol. Biol. Cell* **13**, 854-865.

Fontoura, B. M., Blobel, G., and Matunis, M. J. 1999. A conserved biogenesis pathway for nucleoporins: proteolytic processing of a 186-kilodalton precursor generates Nup98 and the novel nucleoporin, Nup96. *J. Cell Biol.* **144**, 1097-1112.

Franco, M., Chardin, P., Chabre, M., and Paris, S. 1993. Myristoylation is not required for GTP-dependent binding of ADP-ribosylation factor ARF1 to phospholipids. *J. Biol. Chem.* **268**, 24531-24534.

Franco, M., Chardin, P., Chabre, M., and Paris, S. 1995. Myristoylation of ADP-ribosylation factor 1 facilitates nucleotide exchange at physiological Mg^{2+} levels. *J. Biol. Chem.* **270**, 1337-1341.

Franco, M., Chardin, P., Chabre, M., and Paris, S. 1996. Myristoylation-facilitated binding of the G protein $ARF1_{GDP}$ to membrane phospholipids is required for its activation by a soluble nucleotide exchange factor. *J. Biol. Chem.* **271**, 1573-1578.

Franzusoff, A., Redding, K., Crosby, J., Fuller, R. S., and Schekman, R. 1991. Localization of components involved in protein transport and processing through the yeast Golgi apparatus. *J. Cell Biol.* **112**, 27-37.

Franzusoff, A. and Schekman, R. 1989. Functional compartments of the yeast Golgi apparatus are defined by the *sec7* mutation. *EMBO J.* **8**, 2695-2702.

Fries, E. and Rothman, J. E. 1980. Transport of vesicular stomatitis virus glycoprotein in a cell-free extract. *Proc. Natl. Acad. Sci. USA* **77**, 3870-3874.

Garcia-Higuera, I., Gaitatzes, C., Smith, T. F., and Neer, E. J. 1998. Folding a WD repeat propeller. *J. Biol. Chem.* **273**, 9041-9049.

Gaynor, E. C. and Emr, S. D. 1997. COPI-independent anterograde transport: cargo-selective ER to Golgi protein transport in yeast COPI mutants. *J. Cell Biol.* **136**, 789-802.

Gaynor, E. C., Graham, T. R., and Emr, S. D. 1998. COPI in ER/Golgi and intra-Golgi transport: do yeast COPI mutants point the way? *Biochim. Biophys. Acta* **1404**, 33-51.

Gaynor, E. C., te Heesen, S., Graham, T. R., Aebi, M., and Emr, S. D. 1994. Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. J. *Cell Biol.* **127**, 653-665.

Gilstring, C. F., Melin-Larsson, M., and Ljungdahl, P. O. 1999. Shr3p mediates specific COPII coatomer-cargo interactions required for the packaging of amino acid permeases into ER-derived transport vesicles. *Mol. Biol. Cell* **10**, 3549-3565.

Gimeno, R. E., Espenshade, P., and Kaiser, C. A. 1995. *SED4* encodes a yeast endoplasmic reticulum protein that binds Sec16p and participates in vesicle formation. *J. Cell Biol.* **131**, 325-338.

Gimeno, R. E., Espenshade, P., and Kaiser, C. A. 1996. COPII coat subunit interactions: Sec24p and Sec23p bind to adjacent regions of Sec16p. *Mol. Biol. Cell* **7**, 1815-1823.

Granville, D. J., Carthy, C. M., Jiang, H., Shore, G. C., McManus, B. M., and Hunt,

D. W. 1998. Rapid cytochrome *c* release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. *FEBS Lett.* **437**, 5-10.

Hardwick, K. G., Boothroyd, J. C., Rudner, A. D., and Pelham, H. R. 1992. Genes that allow yeast cells to grow in the absence of the HDEL receptor. *EMBO J.* **11**, 4187-4195.

Hardwick, K. G. and Pelham, H. R. 1992. *SED5* encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J. Cell Biol.* **119**, 513-521.

Harlow, E. and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Hartwell, L. H. 1991. Twenty-five years of cell cycle genetics. *Genetics* **129**, 975-980.

Henry, S. A., Atkinson, K. D., Kolat, A. I., and Culbertson, M. R. 1977. Growth and metabolism of inositol-starved *Saccharomyces cerevisiae*. *J. Bacteriol.* **130**, 472-484.

Hicke, L. and Schekman, R. 1989. Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex *in vivo* and *in vitro*. *EMBO J.* **8**, 1677-1684.

Hicke, L., Yoshihisa, T., and Schekman, R. 1992. Sec23p and a novel 105-kDa protein function as a multimeric complex to promote vesicle budding and protein transport from the endoplasmic reticulum. *Mol. Biol. Cell* **3**, 667-676.

Higashio, H., Kimata, Y., Kiriyama, T., Hirata, A., and Kohno, K. 2000. Sfb2p, a yeast protein related to Sec24p, can function as a constituent of COPII coats required for vesicle budding from the endoplasmic reticulum. *J. Biol. Chem.* **275**, 17900-17908.

Hosobuchi, M., Kreis, T., and Schekman, R. 1992. *SEC21* is a gene required for ER to Golgi protein transport that encodes a subunit of a yeast coatomer. *Nature* **360**, 603-605.

Huang, M., Weissman, J. T., Béraud-Dufour, S., Luan, P., Wang, C., Chen, W., Aridor, M., Wilson, I. A., and Balch, W. E. 2001. Crystal structure of Sar1-GDP at 1.7 Å resolution and the role of the NH_2 terminus in ER export. *J. Cell Biol.* **155**, 937-948.

Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. 2003. Global analysis of protein localization in budding yeast. *Nature* **425**, 686-691.

Jackson, M. R., Nilsson, T., and Peterson, P. A. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* **9**, 3153-3162.

Jackson, M. R., Nilsson, T., and Peterson, P. A. 1993. Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* **121**, 317-333.

Jahn, R., Lang, T., and Sudhof, T. C. 2003. Membrane fusion. *Cell* **112**, 519-533.

Jimenez, A. and Davies, J. 1980. Expression of a transposable antibiotic resistance element in *Saccharomyces*. *Nature* **287**, 869-871.

Jones, S., Jedd, G., Kahn, R. A., Franzusoff, A., Bartolini, F., and Segev, N. 1999. Genetic interactions in yeast between Ypt GTPases and Arf guanine nucleotide exchangers. *Genetics* **152**, 1543-1556.

Jämsä, E., Holkeri, H., Vihinen, H., Wikström, M., Simonen, M., Walse, B., Kalkkinen, N., Paakkola, J., and Makarow, M. 1995. Structural features of a polypeptide carrier promoting secretion of a β-lactamase fusion protein in yeast. *Yeast* **11**, 1381-1391.

Jämsä, E., Simonen, M., and Makarow, M. 1994. Selective retention of secretory proteins in the yeast endoplasmic reticulum by treatment of cells with a reducing agent. *Yeast* **10**, 355-370.

Kaiser, C. A., Gimeno, R. E., and Shaywitz, D. A. 1997. Protein secretion, membrane biogenesis, and endocytosis. In J. R. Pringle, J. R.

Broach, and E. W. Jones *The Molecular and Cellular Biology of the Yeast Saccharomyces: Cell cycle and cell biology* (pp. 91-227). New York: Cold Spring Harbor Laboratory Press.

Kaiser, C. A. and Schekman, R. 1990. Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* **61**, 723-733.

Kappeler, F., Klopfenstein, D. R., Foguet, M., Paccaud, J. P., and Hauri, H. P. 1997. The recycling of ERGIC-53 in the early secretory pathway. *J. Biol. Chem.* **272**, 31801-31808.

Kapteyn, J. C., Van Egmond, P., Sievi, E., Van Den Ende, H., Makarow, M., and Klis, F. M. 1999. The contribution of the *O*-glycosylated protein Pir2p/Hsp150 to the construction of the yeast cell wall in wild-type cells and β 1,6-glucan-deficient mutants. *Mol. Microbiol.* **31**, 1835-1844.

Karhinen, L. and Makarow, M. 2004. Activity of recycling Golgi mannosyltransferases in the yeast endoplasmic reticulum. *J. Cell Sci.* **117**, 351-358.

Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M., and Johnson, A. D. 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell* **68**, 709-719.

Kim, H., Melén, K., and von Heijne, G. 2003. Topology models for 37 *Saccharomyces cerevisiae* membrane proteins based on C-terminal reporter fusions and predictions. *J. Biol. Chem.* **278**, 10208-10213.

Kim, W. Y., Cheong, N. E., Je, D. Y., Kim, M. G., Lim, C. O., Bahk, J. D., Cho, M. J., and Lee, S. Y. 1997. The presence of a *Sar1* gene family in *Brassica campestris* that suppresses a yeast vesicular transport mutation *Sec12-1*. *Plant Mol. Biol.* **33**, 1025-1035.

Kimata, Y., Lim, C. R., Kiriyama, T., Nara, A., Hirata, A., and Kohno, K. 1999. Mutation of the yeast ε-COP gene *ANU2* causes abnormal nuclear morphology and defects in intracellular vesicular transport. *Cell Struct. Funct.* **24**, 197-208.

Kuehn, M. J., Herrmann, J. M., and Schekman, R. 1998. COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature* **391**, 187-190.

Kuge, O., Dascher, C., Orci, L., Rowe, T., Amherdt, M., Plutner, H., Ravazzola, M., Tanigawa, G., Rothman, J. E., and Balch, W. E. 1994. Sar1 promotes vesicle budding from the endoplasmic reticulum but not Golgi compartments. *J. Cell Biol.* **125**, 51-65.

Kurihara, T., Hamamoto, S., Gimeno, R. E., Kaiser, C. A., Schekman, R., and Yoshihisa, T. 2000. Sec24p and Iss1p function interchangeably in transport vesicle formation from the endoplasmic reticulum in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **11**, 983-998.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lambert, G., Becker, B., Schreiber, R., Boucherot, A., Reth, M., and Kunzelmann, K. 2001. Control of cystic fibrosis transmembrane conductance regulator expression by BAP31. *J. Biol. Chem.* **276**, 20340-20345.

Lederkremer, G. Z., Cheng, Y., Petre, B. M., Vogan, E., Springer, S., Schekman, R., Walz, T., and Kirchhausen, T. 2001. Structure of the Sec23p/24p and Sec13p/31p complexes of COPII. *Proc. Natl. Acad. Sci. USA* **98**, 10704-10709.

Lehle, L., Cohen, R. E., and Ballou, C. E. 1979. Carbohydrate structure of yeast invertase. *J. Biol. Chem.* **254**, 12209-12218.

Li, D. and Roberts, R. 2001. WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases. *Cell Mol. Life Sci.* **58**, 2085-2097.

Lian, J. P. and Ferro-Novick, S. 1993. Bos1p, an integral membrane protein of the endoplasmic reticulum to Golgi transport vesicles, is required for their fusion competence. *Cell* **73**, 735-745.

Liu, Y. and Barlowe, C. 2002. Analysis of Sec22p in endoplasmic reticulum/Golgi transport reveals cellular redundancy in SNARE protein function. *Mol. Biol. Cell* **13**, 3314-3324.

Ma, D., Zerangue, N., Lin, Y. F., Collins, A., Yu, M., Jan, Y. N., and Jan, L. Y. 2001. Role of ER export signals in controlling surface potassium channel numbers. *Science* **291**, 316-319.

Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S., and Fröhlich, K. U. 2002. A caspase-related protease regulates apoptosis in yeast. *Mol. Cell* **9**, 911-917.

Makarow, M. 1988. Secretion of invertase in mitotic yeast cells. *EMBO J.* **7**, 1475-1482.

Malkus, P., Jiang, F., and Schekman, R. 2002. Concentrative sorting of secretory cargo proteins into COPII-coated vesicles. *J. Cell Biol.* **159**, 915-921.

Martínez-Menárguez, J. A., Geuze, H. J., Slot, J. W., and Klumperman, J. 1999. Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. *Cell* **98**, 81-90.

Marzioch, M., Henthorn, D. C., Herrmann, J. M., Wilson, R., Thomas, D. Y., Bergeron, J. J., Solari, R. C., and Rowley, A. 1999. Erp1p and Erp2p, partners for Emp24p and Erv25p in a yeast p24 complex. *Mol. Biol. Cell* **10**, 1923-1938.

Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S. Y., Hamamoto, S., Schekman, R., and Yeung, T. 1998. COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. *Cell* **93**, 263-275.

Matsuoka, K., Schekman, R., Orci, L., and Heuser, J. E. 2001. Surface structure of the COPII-coated vesicle. *Proc. Natl. Acad. Sci. USA* **98**, 13705-13709.

Miller, E., Antonny, B., Hamamoto, S., and Schekman, R. 2002. Cargo selection into COPII vesicles is driven by the Sec24p subunit. *EMBO J.* **21**, 6105-6113.

Miller, E. A., Beilharz, T. H., Malkus, P. N., Lee, M. C., Hamamoto, S., Orci, L., and Schekman, R. 2003. Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell* **114**, 497-509.

Mizuno, M. and Singer, S. J. 1993. A soluble secretory protein is first concentrated in the endoplasmic reticulum before transfer to the Golgi apparatus. *Proc. Natl. Acad. Sci. USA* **90**, 5732-5736.

Mossessova, E., Bickford, L. C., and Goldberg, J. 2003. SNARE selectivity of the COPII coat. *Cell* **114**, 483-495.

Movafeghi, A., Happel, N., Pimpl, P., Tai, G. H., and Robinson, D. G. 1999. Arabidopsis Sec21p and Sec23p homologs. Probable coat proteins of plant COP-coated vesicles. *Plant Physiol.* **119**, 1437-1446.

Muñiz, M., Nuoffer, C., Hauri, H. P., and Riezman, H. 2000. The Emp24 complex recruits a specific cargo molecule into endoplasmic reticulum-derived vesicles. *J. Cell Biol.* **148**, 925-930.

Määttä, J., Hallikas, O., Welti, S., Hildén, P., Schröder, J., and Kuismanen, E. 2000. Limited caspase cleavage of human BAP31. *FEBS Lett.* **484**, 202-206.

Nakamura, N., Yamazaki, S., Sato, K., Nakano, A., Sakaguchi, M., and Mihara, K. 1998. Identification of potential regulatory elements for the transport of Emp24p. *Mol. Biol. Cell* **9**, 3493-3503.

Nakano, A., Brada, D., and Schekman, R. 1988. A membrane glycoprotein, Sec12p, required for protein transport from the endoplasmic reticulum to the Golgi apparatus in yeast. *J. Cell Biol.* **107**, 851-863.

Nakano, A. and Muramatsu, M. 1989. A novel GTP-binding protein, Sar1p, is involved

in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* **109**, 2677-2691.

Newman, A. P., Shim, J., and Ferro-Novick, S. 1990. *BET1*, *BOS1*, and *SEC22* are members of a group of interacting yeast genes required for transport from the endoplasmic reticulum to the Golgi complex. *Mol. Cell. Biol.* **10**, 3405-3414.

Ng, D. T., Brown, J. D., and Walter, P. 1996. Signal sequences specify the targeting route to the endoplasmic reticulum membrane. *J. Cell Biol.* **134**, 269-278.

Ng, F. W., Nguyen, M., Kwan, T., Branton, P. E., Nicholson, D. W., Cromlish, J. A., and Shore, G. C. 1997. p28 Bap31, a Bcl-2/Bcl-X_L- and procaspase-8-associated protein in the endoplasmic reticulum. *J. Cell. Biol.* **139**, 327-338.

Ng, F. W. and Shore, G. C. 1998. Bcl-X_L cooperatively associates with the Bap31 complex in the endoplasmic reticulum, dependent on procaspase-8 and Ced-4 adaptor. *J. Cell. Biol.* **273**, 3140-3143.

Nilsson, T., Jackson, M., and Peterson, P. A. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* **58**, 707-718.

Nishikawa, S. and Nakano, A. 1993. Identification of a gene required for membrane protein retention in the early secretory pathway. *Proc. Natl. Acad. Sci. USA* **90**, 8179-8183.

Nishimura, N. and Balch, W. E. 1997. A di-acidic signal required for selective export from the endoplasmic reticulum. *Science* **277**, 556-558.

Normington, K., Kohno, K., Kozutsumi, Y., Gething, M. J., and Sambrook, J. 1989. *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell* **57**, 1223-1236.

Novick, P., Ferro, S., and Schekman, R. 1981. Order of events in the yeast secretory pathway. *Cell* **25**, 461-469.

Novick, P., Field, C., and Schekman, R. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21**, 205-215.

Novick, P. and Schekman, R. 1979. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **76**, 1858-1862.

Nufer, O., Guldbrandsen, S., Degen, M., Kappeler, F., Paccaud, J. P., Tani, K., and Hauri, H. P. 2002. Role of cytoplasmic C-terminal amino acids of membrane proteins in ER export. *J. Cell. Sci.* **115**, 619-628.

Nuoffer, C., Jenö, P., Conzelmann, A., and Riezman, H. 1991. Determinants for glycophospholipid anchoring of the *Saccharomyces cerevisiae GAS1* protein to the plasma membrane. *Mol. Cell. Biol.* **11**, 27-37.

Oka, T. and Nakano, A. 1994. Inhibition of GTP hydrolysis by Sar1p causes accumulation of vesicles that are a functional intermediate of the ER-to-Golgi transport in yeast. *J. Cell Biol.* **124**, 425-434.

Oka, T., Nishikawa, S., and Nakano, A. 1991. Reconstitution of GTP-binding Sar1 protein function in ER to Golgi transport. *J. Cell Biol.* **114**, 671-679.

Orci, L., Glick, B. S., and Rothman, J. E. 1986. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell* **46**, 171-184.

Orci, L., Ravazzola, M., Meda, P., Holcomb, C., Moore, H. P., Hicke, L., and Schekman, R. 1991. Mammalian Sec23p homologue is restricted to the endoplasmic reticulum transitional cytoplasm. *Proc. Natl. Acad. Sci. USA* **88**, 8611-8615.

Paccaud, J. P., Reith, W., Carpentier, J. L., Ravazzola, M., Amherdt, M., Schekman, R., and Orci, L. 1996. Cloning and functional characterization of mammalian homologues of the COPII component Sec23. *Mol. Biol. Cell* **7**, 1535-1546.

Pagano, A., Letourneur, F., Garcia-Estefania, D., Carpentier, J. L., Orci, L., and Paccaud, J. P. 1999. Sec24 proteins and sorting at the endoplasmic reticulum. *J. Biol. Chem.* **274**, 7833-7840.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. *Science* **189**, 347-358.

Parlati, F., McNew, J. A., Fukuda, R., Miller, R., Söllner, T. H., and Rothman, J. E. 2000. Topological restriction of SNARE-dependent membrane fusion. *Nature* **407**, 194-198.

Paunola, E., Qiao, M., Shmelev, A., and Makarow, M. 2001. Inhibition of translocation of β-lactamase into the yeast endoplasmic reticulum by covalently bound benzylpenicillin. *J. Biol. Chem.* **276**, 34553-34559.

Paunola, E., Suntio, T., Jämsä, E., and Makarow, M. 1998. Folding of active β-lactamase in the yeast cytoplasm before translocation into the endoplasmic reticulum. *Mol. Biol. Cell* **9**, 817-827.

Payne, W. E., Kaiser, C. A., Bevis, B. J., Soderholm, J., Fu, D., Sears, I. B., and Glick, B. S. 2000. Isolation of *Pichia pastoris* genes involved in ER-to-Golgi transport. *Yeast* **16**, 979-993.

Peng, R., De Antoni, A., and Gallwitz, D. 2000. Evidence for overlapping and distinct functions in protein transport of coat protein Sec24p family members. *J. Biol. Chem.* **275**, 11521-11528.

Peng, R., Grabowski, R., De Antoni, A., and Gallwitz, D. 1999. Specific interaction of the yeast *cis*-Golgi syntaxin Sed5p and the coat protein complex II component Sec24p of endoplasmic reticulum-derived transport vesicles. *Proc. Natl. Acad. Sci. USA* **96**, 3751-3756.

Powers, J. and Barlowe, C. 2002. Erv14p directs a transmembrane secretory protein into COPII-coated transport vesicles. *Mol. Biol. Cell* **13**, 880-891.

Preuss, D., Mulholland, J., Kaiser, C. A., Orlean, P., Albright, C., Rose, M. D., Robbins, P. W., and Botstein, D. 1991. Structure of the yeast endoplasmic reticulum: localization of ER proteins using immunofluorescence and immunoelectron microscopy. *Yeast* **7**, 891-911.

Pryer, N. K., Salama, N. R., Schekman, R., and Kaiser, C. A. 1993. Cytosolic Sec13p complex is required for vesicle formation from the endoplasmic reticulum in vitro. *J. Cell Biol.* **120**, 865-875.

Rapoport, T. A., Jungnickel, B., and Kutay, U. 1996. Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* **65**, 271-303.

Redding, K., Holcomb, C., and Fuller, R. S. 1991. Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **113**, 527-538.

Rexach, M. F. and Schekman, R. W. 1991. Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.* **114**, 219-229.

Roberg, K. J., Bickel, S., Rowley, N., and Kaiser, C. A. 1997. Control of amino acid permease sorting in the late secretory pathway of *Saccharomyces cerevisiae* by *SEC13*, *LST4*, *LST7* and *LST8*. *Genetics* **147**, 1569-1584.

Roberg, K. J., Crotwell, M., Espenshade, P., Gimeno, R., and Kaiser, C. A. 1999. *LST1* is a *SEC24* homologue used for selective export of the plasma membrane ATPase from the endoplasmic reticulum. *J. Cell Biol.* **145**, 659-672.

Rose, M. D., Misra, L. M., and Vogel, J. P. 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell* **57**, 1211-1221.

Rothblatt, J. A., Deshaies, R. J., Sanders, S. L., Daum, G., and Schekman, R. 1989.

Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* **109**, 2641-2652.

Ruohola, H., Kabcenell, A. K., and Ferro-Novick, S. 1988. Reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex in yeast: the acceptor Golgi compartment is defective in the *sec23* mutant. *J. Cell Biol.* **107**, 1465-1476.

Russo, P., Kalkkinen, N., Sareneva, H., Paakkola, J., and Makarow, M. 1992. A heat shock gene from *Saccharomyces cerevisiae* encoding a secretory glycoprotein. *Proc. Natl. Acad. Sci. USA* **89**, 3671-3675.

Saito, Y., Yamanushi, T., Oka, T., and Nakano, A. 1999. Identification of *SEC12*, *SED4*, truncated *SEC16*, and *EKS1*/*HRD3* as multicopy suppressors of ts mutants of Sar1 GTPase. *J. Biochem.* **125**, 130-137.

Saito-Nakano, Y. and Nakano, A. 2000. Sed4p functions as a positive regulator of Sar1p probably through inhibition of the GTPase activation by Sec23p. *Genes Cells* **5**, 1039-1048.

Salama, N. R., Chuang, J. S., and Schekman, R. W. 1997. *SEC31* encodes an essential component of the COPII coat required for transport vesicle budding from the endoplasmic reticulum. *Mol. Biol. Cell* **8**, 205-217.

Salama, N. R., Yeung, T., and Schekman, R. W. 1993. The Sec13p complex and reconstitution of vesicle budding from the ER with purified cytosolic proteins. *EMBO J.* **12**, 4073-4082.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Sata, M., Donaldson, J. G., Moss, J., and Vaughan, M. 1998. Brefeldin A-inhibited guanine nucleotide-exchange activity of Sec7 domain from yeast Sec7 with yeast and mammalian ADP ribosylation factors. *Proc. Natl. Acad. Sci. USA* **95**, 4204-4208.

Sato, K. and Nakano, A. 2002. Emp47p and its close homolog Emp46p have a tyrosine-containing endoplasmic reticulum exit signal and function in glycoprotein secretion in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **13**, 2518-2532.

Sato, K., Nishikawa, S., and Nakano, A. 1995. Membrane protein retrieval from the Golgi apparatus to the endoplasmic reticulum (ER): characterization of the *RER1* gene product as a component involved in ER localization of Sec12p. *Mol. Biol. Cell* **6**, 1459-1477.

Sato, K., Sato, M., and Nakano, A. 2001. Rer1p, a retrieval receptor for endoplasmic reticulum membrane proteins, is dynamically localized to the Golgi apparatus by coatomer. *J. Cell Biol.* **152**, 935-944.

Sato, M., Sato, K., and Nakano, A. 1996. Endoplasmic reticulum localization of Sec12p is achieved by two mechanisms: Rer1p-dependent retrieval that requires the transmembrane domain and Rer1p-independent retention that involves the cytoplasmic domain. *J. Cell Biol.* **134**, 279-293.

Schekman, R. and Orci, L. 1996. Coat proteins and vesicle budding. *Science* **271**, 1526-1533.

Schröder, S., Schimmöller, F., Singer-Krüger, B., and Riezman, H. 1995. The Golgi-localization of yeast Emp47p depends on its di-lysine motif but is not affected by the *ret1-1* mutation in α-COP. *J. Cell Biol.* **131**, 895-912.

Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R. A., and Rothman, J. E. 1991. ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell* **67**, 239-253.

Shaywitz, D. A., Espenshade, P. J., Gimeno, R. E., and Kaiser, C. A. 1997. COPII subunit

interactions in the assembly of the vesicle coat. *J. Biol. Chem.* **272**, 25413-25416.

Shaywitz, D. A., Orci, L., Ravazzola, M., Swaroop, A., and Kaiser, C. A. 1995. Human SEC13Rp functions in yeast and is located on transport vesicles budding from the endoplasmic reticulum. *J. Cell Biol.* **128**, 769-777.

Shimoni, Y., Kurihara, T., Ravazzola, M., Amherdt, M., Orci, L., and Schekman, R. 2000. Lst1p and Sec24p cooperate in sorting of the plasma membrane ATPase into COPII vesicles in *Saccharomyces cerevisiae*. *J. Cell Biol.* **151**, 973-984.

Shugrue, C. A., Kolen, E. R., Peters, H., Czernik, A., Kaiser, C., Matovcik, L., Hubbard, A. L., and Gorelick, F. 1999. Identification of the putative mammalian orthologue of Sec31P, a component of the COPII coat. *J. Cell. Sci.* **112**, 4547-4556.

Simonen, M., Jämsä, E., and Makarow, M. 1994. The role of the carrier protein and disulfide formation in the folding of β-lactamase fusion proteins in the endoplasmic reticulum of yeast. *J. Biol. Chem.* **269**, 13887-13892.

Siniossoglou, S., Wimmer, C., Rieger, M., Doye, V., Tekotte, H., Weise, C., Emig, S., Segref, A., and Hurt, E. C. 1996. A novel complex of nucleoporins, which includes Sec13p and a Sec13p homolog, is essential for normal nuclear pores. *Cell* **84**, 265-275.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. 1996. Crystal structure of a G_A protein βγ dimer at 2.1 Å resolution. *Nature* **379**, 369-374.

Spiliotis, E. T., Manley, H., Osorio, M., Zúñiga, M. C., and Edidin, M. 2000. Selective export of MHC class I molecules from the ER after their dissociation from TAP. *Immunity* **13**, 841-851.

Springer, S., Chen, E., Duden, R., Marzioch, M., Rowley, A., Hamamoto, S., Merchant, S., and Schekman, R. 2000. The p24 proteins are not essential for vesicular transport in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **97**, 4034-4039.

Springer, S., Spang, A., and Schekman, R. 1999. A primer on vesicle budding. *Cell* **97**, 145-148.

Stenbeck, G., Harter, C., Brecht, A., Herrmann, D., Lottspeich, F., Orci, L., and Wieland, F. T. 1993. β'-COP, a novel subunit of coatomer. *EMBO J.* **12**, 2841-2845.

Stevens, T., Esmon, B., and Schekman, R. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell* **30**, 439-448.

Stinchcombe, J. C., Nomoto, H., Cutler, D. F., and Hopkins, C. R. 1995. Anterograde and retrograde traffic between the rough endoplasmic reticulum and the Golgi complex. *J. Cell Biol.* **131**, 1387-1401.

Suntio, T., Shmelev, A., Lund, M., and Makarow, M. 1999. The sorting determinant guiding Hsp150 to the COPI-independent transport pathway in yeast. *J. Cell. Sci.* **112**, 3889-3898.

Supek, F., Madden, D. T., Hamamoto, S., Orci, L., and Schekman, R. 2002. Sec16p potentiates the action of COPII proteins to bud transport vesicles. *J. Cell Biol.* **158**, 1029-1038.

Swaroop, A., Yang-Feng, T. L., Liu, W., Gieser, L., Barrow, L. L., Chen, K. C., Agarwal, N., Meisler, M. H., and Smith, D. I. 1994. Molecular characterization of a novel human gene, *SEC13R*, related to the yeast secretory pathway gene SEC13, and mapping to a conserved linkage group on human chromosome 3p24-p25 and mouse chromosome 6. *Hum. Mol. Genet.* **3**, 1281-1286.

Tang, B. L., Kausalya, J., Low, D. Y., Lock, M. L., and Hong, W. 1999. A family of mammalian proteins homologous to yeast Sec24p. *Biochem. Biophys. Res. Commun.* **258**, 679-684.

Todorow, Z., Spang, A., Carmack, E., Yates, J., and Schekman, R. 2000. Active recycling of yeast Golgi mannosyltransferase complexes through the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **97**, 13643-13648.

Toikkanen, J., Gatti, E., Takei, K., Saloheimo, M., Olkkonen, V. M., Söderlund, H., De Camilli, P., and Keränen, S. 1996. Yeast protein translocation complex: isolation of two genes *SEB1* and *SEB2* encoding proteins homologous to the Sec61 β subunit. *Yeast* **12**, 425-438.

Townsley, F. M. and Pelham, H. R. 1994. The KKXX signal mediates retrieval of membrane proteins from the Golgi to the ER in yeast. *Eur. J. Cell Biol.* **64**, 211-216.

Vetter, I. R. and Wittinghofer, A. 2001. The guanine nucleotide-binding switch in three dimensions. *Science* **294**, 1299-1304.

Votsmeier, C. and Gallwitz, D. 2001. An acidic sequence of a putative yeast Golgi membrane protein binds COPII and facilitates ER export. *EMBO J.* **20**, 6742-6750.

Wadhwa, R., Kaul, S. C., Komatsu, Y., Ikawa, Y., Sarai, A., and Sugimoto, Y. 1993. Identification and differential expression of yeast *SEC23*-related gene (*Msec23*) in mouse tissues. *FEBS Lett.* **315**, 193-196.

Wall, M. A., Coleman, D. E., Lee, E., Iñiguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. 1995. The structure of the G protein heterotrimer $G_{i\,\alpha\,1}\ \beta_1\ \gamma_2$. *Cell* **83**, 1047-1058.

Waters, M. G., Serafini, T., and Rothman, J. E. 1991. 'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature* **349**, 248-251.

Wuestehube, L. J., Duden, R., Eun, A., Hamamoto, S., Korn, P., Ram, R., and Schekman, R. 1996. New mutants of *Saccharomyces cerevisiae* affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex. *Genetics* **142**, 393-406.

Yoshihisa, T., Barlowe, C., and Schekman, R. 1993. Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science* **259**, 1466-1468.

Zieler, H. A., Walberg, M., and Berg, P. 1995. Suppression of mutations in two *Saccharomyces cerevisiae* genes by the adenovirus E1A protein. *Mol. Cell. Biol.* **15**, 3227-3237.